Naturally occurring immunosuppressive substances in plasma, with special reference to malignant disease

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To Sally, Dean and Kim.

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DECLARATION

I declare that I have composed and written this thesis, and that the work presented here was performed entirely by me unless otherwise acknowledged.

Date: 20/12/87 D. M. A. FRANCIS

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ABBREVIATIONS

A2M	Alpha-2-macroglobulin
A2M-P	Alpha-2-macroglobulin-protease complex
ADCC	Antibody dependent cellular cytotoxicity
AFP	Alpha-fetoprotein
AMC	Arm muscle circumference
APC	Antigen presenting cell
APRP	Acute phase reactant protein
B lymphocyte	Bursa-dependent lymphocyte
BMN	Benign malnourished
BSS	Balanced salt solution
BW	Body weight
BWN	Benign well nourished
CDHR	Cutaneous delayed hypersensitivity reaction
CDL	Complement dependent lysis
CMI	Cell-mediated immunity
CN-SIF	Circulating non-specific immunosuppressive factors
Con A	Concanavalin A
CTL	Cytotoxic T lymphocyte
EBV	Epstein-Barr virus
Fab	Antigen binding fragment of IgG
Fc	Crystallizable fragment of IgG
FFA	Free fatty acids
GMuLV	Gross murine leukaemia virus
IAC	Immuno-specific affinity chromatography
IBW	Ideal body weight
IC	Immune complex

IE	Immuno-electrophoresis
Ig	Immunoglobulin
Ir genes	Immune response genes
MAC	Mid arm circumference
MEM test	Macrophage electrophoretic mobility test
MIF	Macrophage inhibition factor
MLC	Mixed lymphocyte culture
MMI	Macrophage migration inhibition
MMN	Malignant malnourished
MOD-MEM	Modified MEM test
MW	Molecular weight
MWN	Malignant well nourished
NK cell	Natural killer cell
NS	Not significant
NSD	Not significantly different
OFA	Oncofoetal antigen
OSA	Organ specific antigen
PB	Peripheral blood
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PG	Prostaglandin
PHA	Phytohaemagglutinin
PPD	Purified protein derivative of mycobacterium tuberculosis
PSA	Plasma suppressive activity
PUFA	Polyunsaturated fatty acids
PVG/C	Portland-Virol-Glaxo rat

PW	Present weight
PWM	Pokeweed mitogen
RI	Radial immunodiffusion
RPM	Revolutions per minute
SA	Suppressive activity
SBTI	Soybean trypsin inhibitor
T lymphocyte	Thymus dependent lymphocyte
TATA	Tumour associated transplantation antigen
TBB	Tris barbitone buffer
TBNAA	Total body neutron activation analysis
TEEM test	Tanned erythrocyte electrophoretic mobility test
Th	Helper T lymphocyte
TPN	Total parenteral nutrition
Ts	Suppressor T lymphocyte
TSA	Tumour specific antigen
TSF	Triceps skin fold
TSRBC	Tanned sheep red blood cells
UW	Usual weight
WAB	Wistar-Albino-Boots rat

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ABSTRACT OF THESIS (Regulation 7.9)

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Title of Thesis Naturally occurring immunosuppressive substances in plasma, with special reference to malignant disease

Evidence suggests that host immunity has an important bearing on the growth and spread of malignant tumours. Naturally occurring immunosuppressive substances have been demonstrated in the plasma of cancer patients, but the nature and relevance of such factors to the impaired immunity of cancer patients and to tumour behaviour are poorly understood. Cellular immunity is impaired in malnourished subjects also; although nutritional repletion with fat-free intravenous fluids may improve nutritional and immunological parameters, the immunological consequences of using solutions rich in polyunsaturated fatty acids are largely unknown.

The aims of the study were to quantify the lymphocyte suppressive activity of plasma (plasma suppressive activity, PSA) and to identify specific substances associated with suppressive activity in plasma from normal subjects, and patients with benign or malignant diseases and with various degrees of malnutrition. Also, an attempt was made to correlate PSA with tumour behaviour in an animal model, firstly by measuring PSA before, during and after tumour growth, and secondly by observing tumour growth after artificially increasing PSA. In other studies in vitro lymphocyte reactivity was measured firstly after incubation with fat emulsion, and secondly in patients receiving parenteral fat emulsions.

The study found that PSA was low in healthy subjects and patients with benign disease, and was associated entirely with the plasma protease inhibitor alpha-2-macroglobulin (A2M). PSA was raised moderately, although significantly, in malnourished patients with benign disease and correlated significantly with the extent of nutritional impairment; PSA was associated mainly with A2M but also with a small molecular weight peptide fraction. Patients with malignant disease had very high PSA which did not correlate with nutritional impairment and which was associated mainly with A2M but also with immune complexes, IgG, Fc fragments and a small molecular weight peptide fraction. Plasma A2M concentrations did not differ significantly between subject groups although the amount of suppressive activity associated with A2M did. PSA increased with tumour growth in the animal model but returned to pre-tumour levels after complete removal of tumours. Tumour growth was increased significantly in animals in which PSA was elevated at the time of tumour transplantation. Intravenous fluids containing fat significantly depressed in vitro lymphocyte reactivity in the laboratory and clinical studies.

The study shows that A2M is an important regulator of lymphocyte reactivity in health and in malignant disease, and demonstrates a mechanism of immunological evasion by tumours by way of naturally occurring immunoregulatory plasma factors.

INTRODUCTION

The thesis examines the role of naturally occurring immunosuppressive substances, mainly in relation to malignant disease but also in malnutrition. Members of the laboratory in which the work was performed had undertaken studies of naturally occurring lymphocyte suppressive activity in relation to organ transplantation and, as the author had interests in surgical oncology and immunology, attention was directed towards investigating suppressive substances in cancer patients.

It appeared to the author that a paradox existed in the interaction of tumours with their hosts. On the one hand, the literature indicated that an immunologically competent subject could respond specifically to new or foreign antigenic material and reject it, and that tumour-bearing patients and experimental animals frequently exhibited specific immune responses to tumours or tumour extracts. On the other hand, it was obvious that cancers grew progressively, even in patients in whom specific immune responses could be demonstrated. Thus, it appeared that tumours had found a way of evading either detection or destruction by the hosts' immune responses, a concept known as "immunological escape". This thesis considers the part played by naturally occurring plasma factors in the immunological escape of tumours.

The experimental work involved four distinct stages. First, lymphocyte reactivity and the ability of plasma to suppress lymphocyte reactivity was measured and quantified in three groups of subjects - healthy volunteers and hospitalised patients with benign or malignant disease. The influence of nutritional status had to be taken into account when the results were analysed. Second, plasma from a small number of subjects from each of five experimental groups - healthy volunteers, benign

well nourished, benign malnourished, malignant well nourished and malignant malnourished subjects - was analysed in detail with the aim of identifying the substances responsible for suppressive activity and assessing the relative contribution of each substance. Third, experiments were undertaken to assess the effects on lymphocyte reactivity of solutions used for parenteral nutrition in malnourished patients. Fourth, an animal model was used in an attempt to correlate tumour growth with lymphocyte suppressive activity in plasma and to observe tumour growth when suppressive activity was increased by means unrelated to tumour activity.

The thesis is presented in six chapters. The literature review (Chapter 1) examines the immune response and its control in relation to anti-tumour mechanisms, and reviews the principal, currently recognised ways by which tumours evade immune attack. The biological role of the plasma protease inhibitor alpha-2-macroglobulin as a non-specific inhibitory substance is reviewed and hypotheses are proposed concerning alpha-2-macroglobulin in cancer patients. A review of some aspects of the literature on malnutrition identifies the nutritional deficiencies which are present in many cancer patients, highlights the wide-ranging consequences of malnutrition on the immune system and critically assesses current methods of nutritional assessment. Although the literature indicates that nutritional repletion of malnourished individuals can reverse many of their immunological deficiencies, there is very little data concerning the immunological effects of fluids used for parenteral nutrition; this lack of information provided the stimulus for studies of parenteral feeding solutions.

The methods used for the experimental work are presented in chapter two; they include the Tanned Erythrocyte Electrophoretic Mobility (TEEM)

test, gel filtration chromatography, single- and fused-rocket immuno-electrophoresis, immuno-specific affinity chromatography, a polyethylene glycol precipitation technique and radial immunodiffusion. The background, development and method of the TEEM test are presented, because the test is not used widely at present and has been misrepresented and misinterpreted in the past. The methods of nutritional assessment are presented.

The results are presented in chapters three, four and five. Chapter three contains the results of studies of lymphocyte reactivity and plasma suppressive activity, and the influence of nutritional status and the site and stage of malignant diseases on these two parameters. The results of experiments with plasma fractions and individual suppressive substances are also presented. The studies of the effects of nutritional fluids on lymphocyte reactivity and the results of lymphocyte reactivity in patients receiving parenteral nutrition are presented in chapter four. The results of tumour growth studies in animals are given in chapter five.

The results of most of the experiments are discussed individually within appropriate sections. The experimental work is discussed as a whole, and in relation to the reviewed literature and the author's hypotheses, in chapter six. A further mechanism of immunological escape is proposed and an explanation is offered for the apparent paradox of tumour growth in the presence of specific anti-tumour immune responses. A list of publications, which contain some contribution from the thesis, is shown after the references.

CHAPTER 1

LITERATURE REVIEW

1.1 DEFINITIONS OR DESCRIPTIONS

It is often thought that cancer is a disease of modern times but malignant disease has affected mankind from the time of his first appearance on earth. Pithecanthropus, the Java 'ape-man' discovered in 1891 and believed to be about half a million years old, had a tumourous growth on his femur. Ancient manuscripts from Egypt and India, written centuries before Christ, describe ulcerating tumours, and the early Greek physicians, including Hippocrates, wrote of men being plagued by a variety of growths with peculiar features. Galen wrote a treatise on tumours and classified them as benign or malignant. As humans now live considerably longer than in former times and as malignant disease is generally more common with increasing age, cancer has now become the second commonest cause of death in the western world.

Although cancer has been studied extensively, an entirely satisfactory definition of malignancy has yet to be formulated. Many 'definitions' of cancer are in fact observations or descriptions of the behaviour of neoplastic cells, either grouped together as solid tumours or disseminated as haematological malignancies.

Malignant tumours have several characteristic features. Firstly, the growth of constituent cells is not under the same control as that of normal tissues, with the result that growth is disorganised and uncoordinated. Secondly, malignant cells have the ability to invade host tissues by direct extension and dissemination through the circulation, lymphatic channels and across body cavities to sites unconnected with the primary growth. Uncontrolled growth and spread impairs normal function of involved tissues by biochemical or mechanical means and eventually kills the host.

1.1.1 TRANSFORMATION AND EVASION

The development and progression of cancer involves two fundamental processes, namely transformation and evasion. Transformation removes the growth-limiting restrictions from a cell so that it continues to divide and produce generations of cells similarly free of growth limitations. If transformation was all that was required for a cell to grow successfully as a neoplastic cell then it must be a rare event, considering the rate of cell turnover in the body in comparison with the incidence of cancer. Cell marker studies indicate that experimental carcinogens transform normal cells with a frequency several orders of magnitude higher than the observed rate of overt tumour development. Thus, it appears that not all transformed cells survive to produce clinically detectable neoplasms.

One explanation for the discrepancy between rates of transformation and cancer occurrence is that the human immune system is able to prevent transformed cells from becoming overt tumours. Burnet[1,2], in 1957, proposed the concept of "immune surveillance" whereby cells of the host's immune system detect and destroy 'foreign' malignant cells. Burnet's theory has been debated intensively by protagonists[3-6] and antagonists[7-11], and has not received universal acceptance. If transformation is not a rare event, then anti-tumour mechanisms must be highly effective in eliminating transformed cells early in their natural history. At present, it is not possible to measure the success of anti-tumour mechanisms as there is no way of detecting <u>in vivo</u> when the growth and reproduction of transformed cells is overcome. It is evident that many transformed cells are not eliminated and undergo the second critical process of evasion.

Evasion allows transformed cells to resist destruction by immune responses of the host and, as a consequence, transformed cells continue

their pattern of abnormal growth and a malignant tumour is formed. In spite of an enormous amount of study, the precise role of the host's immune system in the development, control and progression of malignant tumours is unclear. Many tumours in humans and experimental animals are able to stimulate specific immune responses within their hosts and, although such responses may retard the growth and dissemination of the tumour by killing malignant cells, the tumour is still able to proliferate and overcome the host.

1.2 THE IMMUNE RESPONSE

The human immune system is a highly complex, integrated homeostatic mechanism of defence against foreign material which may threaten the integrity of the host. It has non-specific and specific components. Non-specific or innate components include mechanical barriers to pathogens (skin, mucous membranes), bacteriocidal substances in tissues and body fluids (lysozyme, complement, basic peptides), and the process of phagocytosis or entrapment within circulating phagocytic cells (monocytes) or tissue phagocytes (macrophages). Once a foreign antigen has penetrated or by-passed these first-line defences it comes into contact with cells of the immune system, whereupon specific immune responses occur. Two different specific responses may arise: (a) the humoral response: production of antibodies by plasma cells derived from bursa-dependent (B) lymphocytes, and (b) the cell-mediated response: production of specifically sensitised lymphoid cells processed by or dependent upon the thymus (T lymphocytes) to become either effector cells of cell-mediated immunity or regulators of other immune cells.

Initiation of an immune response requires the interaction of

antigen with an antigen presenting cell (APC) of the macrophage series. Macrophages act as accessory cells in the immune response and are essential for the activation of T lymphocytes; their function is initial binding and presentation of antigen to lymphocytes and regulation of lymphocyte responsiveness by secretion of suppressive and enhancing factors[12].

Processing of antigens by APC is required for optimal antigenicity and may influence the nature of the induced immune response[13-15]. Physical interaction between antigen-bearing macrophages and lymphocytes is required for functional differentiation and maturation of lymphocytes during an immune response, and requires that the cells have their major histocompatibility complex (MHC) antigens in common. The APC cell membrane is a site of expression of histocompatibility linked immune response (Ir) genes which regulate T cell activity[16,17]. Antigens are presented to T cells in some structural or functional association with antigenic determinants encoded by Ir genes (Ia determinants or class II MHC molecules) on the cell surface of the macrophage. T lymphocytes then recognise the antigen - Ia complex by way of antigen-specific membrane receptors, some of which carry determinants coded by MHC genes[18]. Following antigen recognition, T cells become primed. An antibody response may occur if B lymphocytes have already bound the antigen to their surface receptors and have produced the "first signal" which has led to inactivation of the cell (i.e. tolerance); interaction and co-operation between primed T cells and B cells produces the "second" or activating signal[19]. After antigenic stimulation, differentiating plasmablasts and plasma cells appear within lymphoid tissue. Alternatively, a cell-mediated response occurs if the T cell is activated by interaction with the macrophage-associated antigen and a signal is passed to the cell nucleus so that lymphocyte transformation occurs. Although the "two signal"

model[19,20] is not accepted universally, it serves as a useful schema for understanding cellular interactions and co-operation in the immune response to antigen.

Cell-mediated immune responses are brought about by two effector mechanisms: firstly, by the release from lymphocytes of biologically active substances called lymphokines[21,22] which modulate activities of other cells involved in immune and inflammatory responses, and secondly, by the generation of lymphoid cells with cytotoxic ability.

1.2.1 LYMPHOKINES

Lymphokines are produced by T and B cells and make an important contribution to the efferent limb of the immune response by local amplification of the sequelae of lymphocyte-antigen interaction. Rocklin[23] has estimated that less than 5% of the cellular infiltrate of a cutaneous delayed hypersensitivity reaction (CDHR) is sensitised specifically to the antigen initiating the reaction, the remainder of infiltrating mononuclear cells being recruited non-specifically by lymphokines released from sensitised lymphocytes. Lymphokines also participate in local regulation of immunological responses as some have helper and suppressor activities and others exert a local negative feedback effect to prevent excessive production of lymphokines[24].

Plasma protease inhibitors (Section 1.5.8) have an important <u>in vivo</u> function in the regulation of immune responses by complex interaction with lymphokines and lymphokine inhibitors[25,26].

1.2.2 CELL-MEDIATED CYTOTOXICITY

Cell-mediated cytotoxicity refers to lysis of targets by one of

three mechanisms which involve specialised effector cells: (a) T lymphocyte cytotoxicity (CTL), (b) antibody-dependent cellular cytotoxicity (ADCC), and (c) cytotoxicity exhibited by activated macrophages.

Generation of cytotoxic T lymphocytes (CTL) with antigenic specificity occurs after interaction of a T lymphocyte with an antigenic determinant associated with MHC antigens[27]. Unlike ADCC, target cell killing by CTL is a cyclic phenomenon in as much as a single CTL has the capacity to lyse more than one target cell[28]. Expression of T cell cytotoxicity is restricted to lymphocytes bearing MHC antigens shared or cross-reacting with sensitising cells. CTL can be induced in vitro in response to viral, xenogeneic and modified "self" antigens recognised in association with MHC antigens, which provide the primary stimulus for CTL when complexed with "foreign" antigenic determinants[29].

1.3 REGULATION OF THE IMMUNE RESPONSE

The immune system depends on sophisticated regulatory mechanisms for maintenance of its functional integrity. The major factors influencing or regulating immune responses include host genetic factors, lymphocytes with suppressor functions and anti-idiotypic networks. Non-specific immunoregulatory factors and changes in antigenicity have an important bearing on immune responsiveness and are discussed in the section dealing with the escape of tumours from immune destruction (Section 1.5).

1.3.1 GENETIC CONTROL

Immune responsiveness is controlled by Ir genes, the products of which act by influencing the size and specificity of an immune response. Some Ir genes act non-specifically, as if setting the "background tone" of responsiveness, while others influence the host's response to specific

antigens[30]. Genes controlling antibody responses to a variety of synthetic and naturally occurring proteins are linked by chromosomal positioning of genes with the MHC[31]. MHC linkage of Ir genes may be observed when the dose of stimulating antigen is sufficiently low for only one immuno-dominant determinant to be recognised. As the response to each determinant of a complex antigen is controlled by separate Ir genes, it is unlikely that the various antigenic determinants would be associated consistently with either all high or all low responder Ir genes, but rather a mixture of high and low responses would be expected[31]. Therefore, genetic control becomes an important influence on the host's ability to launch an effective response against an antigen with multiple epitopes (such as a tumour cell) if, for example, the individual has predominantly weak responses to the complex antigen[32].

1.3.2 SUPPRESSOR T LYMPHOCYTES

Human T lymphocytes with a suppressor function (suppressor T cells - Ts) can be distinguished from other T and B lymphocytes by surface phenotype and biological properties. Membrane determinants of Ts include the presence of Fc receptors, and differentiation antigens detected by monoclonal antibodies (e.g., OKT8) or heterologous antisera. T cell-mediated immune suppression performs at two functionally distinct levels: (a) antigen specific or non-specific suppression, which is dependent on exposure of host cells to antigens or mitogens respectively, and (b) idiotypic suppression, which is brought about by interaction of lymphocytes with idiotypic determinants on antibody molecules formed specifically in response to antigens. This second suppressive network has the advantage that the anti-idiotype specific Ts induced by the

immunoglobulin molecule can still exert control of immune reactivity after the antigen has been eliminated and after the specific antigen-induced Ts cell activity has declined.

Long-lived memory suppressor cells are produced in response to antigen exposure so that subsequent exposure leads to an augmented suppressor response which is important in the maintenance of tolerance and immune homeostasis[33]. Suppressor T cells are involved in regulation of a wide range of immune responses including antibody production[34], expression of delayed hypersensitivity[35], induction of tolerance in humoral and cell-mediated responses[36], induction of suppression by the mitogen Concanavalin A (Con A)[37], suppression of immune responses in neonates[38] and the elderly[39], and suppression to self antigens[40,41]. Because of the far reaching influences of Ts in immune regulation and tolerance, it is not surprising that imbalances of Ts activity have been associated with several disease states. Manipulation of the immune system through T cell suppression could be a possible mechanism by which a tumour could evade destruction by the host. The possible role of Ts in tumour evasion is discussed in Section 6.1.

1.3.3 ANTI-IDIOTYPIC NETWORKS

The network model of anti-idiotype responses as proposed by Jerne[42] in 1974 provides a theoretical model of immune regulation by antibody-cell interactions. Following contact with antigen, antibodies are formed with common specificity but with different antigen binding sites ("idiotypes"). B lymphocyte cell surface receptors have the same idiotype as the immunoglobulin which they secrete. The network theory states that the host has the ability to respond to these idiotypes ("auto-anti-idiotype" responses) and to form antibodies against them ("anti-idiotypic"

antibodies). Anti-idiotypic antibodies can inhibit formation of antibody-forming cells and suppress the generation of antibody or cell-mediated responses. Suppression or enhancement is brought about by binding of the anti-idiotypic antibody to the antigen-specific cell surface receptor ("idiotype"). Thus, the idiotype can act as either an antibody by combining with the antigenic determinants of the antigen molecule ("epitope"), or as an antigen by combining with the anti-idiotype.

While there is no doubt that auto-anti-idiotypic responses play a significant role in immune regulation, tolerance and auto-immunity, evidence is awaited concerning the role of anti-idiotypic networks in the development or progression of transformed cells and clinical malignancy.

1.4 THE IMMUNE RESPONSE AGAINST CANCER

1.4.1 TUMOUR ANTIGENS

A tumour antigen is any molecular arrangement present within or on a malignant cell but not in healthy cells of the same original tissue at the same stage of development of that tissue. Four different species of tumour antigens have been classified: (1) tumour specific antigens (TSA), a rare class of antigens found only on malignant cells, (2) organ specific antigens (OSA), cross reacting antigens from tumours derived from common tissues. It is uncertain whether OSA are truly organ specific or are in fact (3) oncofoetal antigens (OFA), which are synthesised by a limited number of cells in embryonic tissues and are either absent from healthy adult cells or present at low concentrations. (4) Tumour associated transplantation antigens (TATA) are located on the surface of tumour cells and are capable of protecting pre-immunised animals against subsequent challenge with the same tumour; most TATA are not specific for malignant

cell clones and may be detected on embryonic cells and cells infected with but not transformed by oncogenic viruses, and so these antigens are better regarded as "tumour-associated" rather than "tumour-specific" transplantation antigens[43].

Identification of the precise nature of tumour surface and intracellular antigens is not only of academic interest. Malignant tumours have been regarded as variants of organ allografts[44] and in some respects a parallel may exist between allograft rejection and tumour elimination. However, allografts are rejected universally from unmodified recipients while malignant tumours frequently progress in spite of specific immunological reactions by the host, indicating that the immunogenicity of the two tissues is different. Altered antigenicity of tumour cells may be one factor contributing to their immunological escape (Section 1.5.3).

Several biological functions have been ascribed to tumour antigens (reviewed by Ristow[45]). Tumour antigens may represent important receptor sites on the cell surface or may have enzymatic activity which influences the malignant potential of the tumour. Haywood et al[46] found an inverse relationship between the frequency of normal transplantation antigens (H-2) and tumour specific antigens on the surface of cells transformed by methylcholanthrene in syngeneic mice: tumours which were highly immunogenic had quantitatively less H-2 antigen on their cell surfaces and vice versa, and early lung metastases were associated with low levels of tumour specific immunogenicity and high levels of H-2 antigenicity. These observations suggest that tumour specific antigens play a role in the tumour-host relationship in that the highly immunogenic tumours remained localised while tumours of low immunogenicity were able to metastasise. Thus, surface antigens on tumour cells may be important determinants of the malignant potential of a tumour and, by implication, the success or

failure of the host's immune response in arresting tumour spread.

The existence of tumour antigens distinct from surface molecules of normal cells suggests that it is reasonable to expect specific tumour-directed immune responses in subjects bearing malignant tumours.

1.4.2 ANTIBODY RESPONSES TO TUMOURS

1.4.2 (a) Circulating antibodies

Anti-tumour antibodies have been detected in the sera of many patients with different malignant diseases. Antibodies directed against intracellular antigenic constituents are of uncertain significance; other antibodies, directed against tumour cell surface components, may be formed in response to TSA or TATA. Reactions of cancer sera against allogeneic tumours of similar histogenesis could be due to antibodies formed against TATA, more than one of which may be expressed by a given tumour cell[43].

The situation is confused by the presence of "anti-tumour" antibodies (or at least antibodies cross-reacting with tumour cell lines) in apparently healthy individuals. Edynak et al[47] detected antibodies directed against <u>in vitro</u> cultured allogeneic breast cancer cells in 22 of 24 patients with early breast carcinoma but also in 4 of 20 age and parity-matched healthy individuals. The antibodies detected in healthy controls could have been allo-antibodies cross-reacting with TATA as no consideration was given to histocompatibility differences. Also, it is conceivable that some subjects had overcome and eliminated transformed malignant cells and that circulating antibodies were the remaining evidence of a successful immunological challenge.

Some studies have found higher titres of antitumour antibodies in patients with smaller tumours and have noted a decline in antibody titres

with advanced malignancy. This may be due to adsorption and sequestration of antibody by large tumours, or decreased antibody production as a result of antigenic modulation (Section 1.5.3), or general impairment of immune function with advanced malignancy[48]. Alternatively, anti-idiotypic antibodies could combine with anti-tumour antibodies so that they are no longer detectable as freely circulating antibody[49]. Anti-idiotypic antibodies have been identified in some patients with malignancy: anti-Ig-Fc antibodies have been isolated from tumours and serum of patients with melanoma and carcinoma of the lung, tongue, colon, rectum, skin and breast[50], and anti-Fc and anti-Fab antibodies have been detected in females with breast carcinoma[51]. The biological significance of anti-idiotypic antibodies in cancer patients is uncertain but theoretically they could result in suppression of responses against tumour antigens or induce tolerance towards them (Section 1.3.3).

1.4.2 (b) Tumour-associated antibodies

Immunoglobulins can be demonstrated also within solid tumours. Tumour-associated antibodies are usually of the IgG class in experimental animals while several classes have been detected in human tumours. Roberts et al[52] identified IgG and IgA in all extracts from malignant breast tumours in 55 women but demonstrated IgM in only 19 of the 55 extracts. Levels of IgG in these malignant tumours correlated with the intensity of round cell and plasma cell infiltrate[52], suggesting that the antibodies were synthesised <u>in situ</u>. Indeed, antibody production within tumours <u>in vitro</u> has been demonstrated by Hurlimann et al[53] in cultures of human breast cancer.

1.4.2 (c) The role of anti-tumour antibodies

The obvious role of "antitumour" or tumour-associated antibodies is destruction of tumour cells, and several mechanisms have been identified by which tumour cells can be eliminated by antibodies.

Firstly, Ran et al[54] demonstrated complement dependent lysis (CDL) of tumour cells: their syngeneic polyoma virus-induced sarcoma (SEYF - a) stimulated a strong antibody response in a murine host and antibody localised on tumour cells; addition of exogenous complement resulted in tumour cell lysis. Although CDL is effective in vitro, its relevance in vivo has been questioned[46], especially in species (such as rodents) in which the complement system is developed poorly.

Secondly, specific antibodies can co-operate with macrophages, polymorphonuclear leukocytes and mononuclear "killer" cells from non-immunised donors to bring about lysis of a target cell, a phenomenon called antibody dependent cell-mediated cytotoxicity (ADCC)[55]. All of these lymphoreticular cells have Fc receptors. ADCC occurs when specific IgG attaches to appropriate antigenic determinants (epitopes) of the target cell by the Fab component; the non-sensitised effector cell attaches to the intact Fc portion of the IgG molecule which effectively bridges the effector and target cells.

Thirdly, tumour cell destruction can be achieved by macrophages as a result of opsonisation by specific antibody[56]. Haskill et al[57] showed that dispersed cells from the murine T1699 spontaneous mammary adenocarcinoma were destroyed in vitro by host macrophages whereas tumour cells from mice with low antibody titres were not destroyed; the difference in killing was attributed to greatly reduced amounts of tumour-associated antibody in the latter group.

Antibodies adversely affect tumour cells in ways other than cell

lysis. Binding of antibody may result in inhibition of cell division[46]. The inhibitory effect of antibody on the amoeboid-like movement of tumour cells could play a significant role in preventing metastases by inhibiting migration of tumour cells into blood and lymphatic channels[46].

In summary, tumour-bearing hosts are capable of producing specific anti-tumour antibodies which are often associated closely with the constituent cells of a tumour. Although mechanisms exist whereby antibody reactions are able to destroy tumour cells, it is clear that in many cases antibody responses are weak and tumours continue to grow in the presence of specific antibody responses.

1.4.3 CELL-MEDIATED RESPONSES TO TUMOURS

Resistance to syngeneic tumour cells in experimental animals is predominantly a cell-associated reaction of the delayed hypersensitivity type which can be transferred adoptively by lymphoid cells but not with serum[10]. Therefore, cell-mediated immunity (CMI) may be of greater importance than humoral responses in the overall response against cancer.

1.4.3 (a) Studies of <u>in vivo</u> and <u>in vitro</u> CMI

Two main approaches have been followed in the study of cellular immune responses against cancer: firstly, demonstration of host cells sensitised to tumour cells, tumour extracts or soluble "tumour antigens", and secondly, demonstration of the ability of host lymphoid cells to destroy viable tumour cells.

Cutaneous delayed hypersensitivity reactions (CDHR) to autologous and allogeneic tumours have been interpreted as evidence of anti-tumour CMI in cancer patients. The subject of CDHR in cancer patients has been

reviewed recently by Heberman[58]. Although techniques for performing CDHR testing can be standardised, interpretation of the results is often difficult, especially in malnourished cancer patients[59]. The significance of CDHR in terms of tumour immunity has not been established satisfactorily.

Cancer patients frequently fail to show CDHR to extracts of their own tumours and positive CDHR have been observed in patients with malignancy of tissues quite different from those used to prepare the challenge tumour extract[60]. Also, some healthy individuals produce positive CDHR when challenged with tumour cell membranes[60]. There are many methodological problems associated with skin testing and these are compounded by the heterogeneity of the "antigen mixture" used as the injected stimulus. Tumours bear many different antigens, including some MHC products, and a positive CDHR to tumour extracts may represent a cross reaction to TATA rather than TSA. Failure to detect a CDHR to a tumour extract may simply reflect impairment of the subject's ability to launch an inflammatory response, or, perhaps of much greater significance, that the tumour had successfully evaded detection by the immune system. Alternatively, the tumour may have been recognised by the host but the effector limb of the response had been inhibited. Thus, the value of CDHR to the analysis of immune responses against cancer, as well as in the diagnosis and management of cancer patients, is limited.

Many <u>in vitro</u> studies of CMI have been undertaken to detect lymphocytes which are sensitised specifically to tumours or capable of mediating tumour cell destruction[10,58,61]. Peripheral blood lymphocytes (PBL) from the majority of cancer patients show some evidence of sensitisation to autologous and allogeneic tumour tissue, as detected by lymphocyte transformation, macrophage migration inhibition or

electrophoretic mobility tests. Similar reactions are found in a minority of apparently healthy subjects, an observation which casts doubt on the specificity of the reaction and possibly the validity of the control subjects. Some studies have demonstrated correlations between the degree of lymphocyte sensitivity and the extent of disease: generally, patients with advanced malignancy exhibit either weaker responses or a lower rate of positive lymphocyte responses to tumour extracts than patients with early disease. In this respect, the findings of Singh et al[89] are typical: in a study of several immune parameters in 38 patients with advanced upper gastrointestinal carcinoma, they found severe depression of absolute and T lymphocyte counts, depression of CDHR to purified protein derivative of Mycobacterium tuberculosis (PPD) and phytohaemagglutinin (PHA), and depressed IgG levels, and found no correlation of these immune parameters with extent or primary site of disease, the performance status of the patients, or their response to combination chemotherapy. There was wide overlap between the results of various subgroups of patients and such poor specificity that the parameters could not be used as prognostic indicators. One frustrating aspect of this and other similar studies[90-93] is that it is impossible to state whether the impairment of immune parameters was a cause or an effect of the progression of the malignant process. It is well recognised that immune function may be impaired by malnutrition, surgery, anaesthesia, chemotherapy and radiotherapy, factors which are associated frequently with malignant disease. However, a generalised depression of the host's immune response could be a mechanism by which tumours escape from detection or destruction by the immune system, a theme discussed later in Section 1.6.

Some <u>in vitro</u> tests of tumour-directed CMI have been proposed as

methods of diagnosis of pre-clinical or occult malignancy[62,63]. In spite of initial enthusiasm, such tests have not received general acceptance.

In summary, considerable research effort has been spent investigating cellular responses to tumours. Although many patients launch specific tumour-directed responses involving lymphoid cells, clinical observation indicates that such responses only very rarely rid the host of a tumour and are depressed in most cancer patients.

1.4.3 (b) Local cell-mediated responses

Russell[64] suggested in 1908 that inflammatory cell infiltrates seen in tumours were an indication of host resistance. Subsequently, many authors have studied the nature and activity of host cells within tumours with the aim of establishing correlations between qualitative or quantitative aspects of tumour infiltrates and tumour behaviour.

Cellular infiltrates in tumours have been correlated with the histological type and degree of tumour cell differentiation. Ioachim et al[65] found that well differentiated squamous cell carcinomas of the lung contained significantly greater numbers of infiltrating cells than did poorly differentiated or undifferentiated tumours, the latter being almost devoid of host cells; lymphocytes were often seen between and even within tumour cells in heavily infiltrated tumours. Electron microscopy has shown tumour cells in intimate contact with infiltrating macrophages and lymphocytes, which exhibit interdigitating surface microvilli characteristic of cytotoxic T lymphocytes[66] (Section 1.2.2). Plasma cells have been demonstrated within well differentiated keratin-forming squamous cell carcinomas of the lung but only infrequently in other types of lung neoplasms[67], and similar associations have been found in squamous cell carcinomas of the skin, oesophagus and cervix.

Intense lymphocytic infiltration has been observed around premalignant lesions and in situ carcinomas of the bronchus, cervix, vagina and skin, and the degree of cellular infiltrate in these early lesions was nearly always greater than in advanced cancers or their metastases[6]. Thus, the immune response appears stronger (or certainly more evident) in small tumours than in more advanced tumours, perhaps because smaller lesions are more immunogenic and the reactive capacity of the host is greater when the tumour is small[6].

Lauder et al[68] investigated lymphocytic infiltration in 23 cases of neuroblastoma and demonstrated a good correlation with survival, although an inverse relationship was observed between plasma cell infiltrate and a good prognosis. The presence of metastases did not invalidate the association of lymphocytic infiltration with a favourable outcome, suggesting that local cellular reactions may reflect the overall efficiency of the host's response to malignant cells. Other studies have shown similar results. Cochran[69], for example, concluded from histological studies of 165 patients with malignant melanomas that the presence of lymphocytes and absence of plasma cells in tumours were important determinants of a good prognosis.

Further circumstantial evidence of the role of cell-mediated immunity in tumour "rejection" comes from histological studies of regressing tumours. Histologically, tumour regression is always accompanied by a heavy local lymphoid infiltrate; lymphocytes and macrophages surround tumour cells which are later destroyed and, as the tumour reduces in size, healing by fibrosis takes place[70]. However, by time the primary tumour has disappeared, metastases may have occurred and these are not subject to the same host responses as the primary tumour[71].

1.4.3 (c) The role of infiltrating cells

Many cells of host origin found within tumours are immunologically active and have been recruited specifically. Klein et al[72] showed that infiltrating T lymphocytes from a variety of human malignant tumours were metabolically active and had significantly higher rates of DNA synthesis than PBL, indicating that they were not simply "resting" cells. Plata et al[73,74] studied cellular infiltrates in murine Moloney sarcoma virus (MSV)-induced tumours (highly antigenic tumours which are rejected usually unless the host is immunosuppressed); the in vitro cytolytic activity of intratumour lymphoid cells was maximal at the onset of tumour regression and removal of macrophages by treatment of tumour-derived leukocytes with carbonyl iron powder did not reduce the overall leukocyte cytolytic activity. After removal of T lymphocytes by anti-Thy 1.2 serum and complement, there was a sharp drop in cytolytic activity although some remained, presumably due to natural killer cell activity.

In comparative studies of regressing and progressing Moloney sarcomas in mice, Russell et al[75] and Gillespie et al[76] found that regressing neoplasms contained relatively more T lymphocytes distributed throughout the tumour mass, whereas the T cell content of progressing sarcomas was lower and T cells were confined to the peripheries of tumours.

There is now considerable evidence that macrophages are major effector cells in both specific and non-specific cell-mediated cytotoxicity[77]. Studies in the rat[78] and with human breast tumours and melanomas[79] suggest that the activity of phagocytic cells influences the distribution of metastases. Macrophages participate in tumour cell destruction by two mechanisms: (a) macrophage activation[80-82] (conversion of a normal macrophage into a cell exhibiting non-specific cytotoxicity for tumour cells), and (b) macrophage arming[83-85] (functionally distinct

cells that are dependent on lymphocytes and kill in an immunologically specific manner).

Within the last 15 years it has been observed that lymphoid cells from normal animals and humans not previously exposed to tumour cells or antigens have significant levels of cytotoxic reactivity against syngeneic and allogeneic tumour cells. Such natural or "non-immune" cytotoxicity has been attributed to natural killer (NK) cell activity. Many features of NK cells have yet to be elucidated but it is generally accepted that NK cells have significant antitumour potential[86].

Fc receptors have been demonstrated on the surface of NK cells and, while the role of Fc receptors in these cells is uncertain, NK activity can be suppressed by immunoglobulins and immune complexes binding by these receptors and causing steric interference of cell to cell contact[87]. NK cell activity can be reduced greatly by treatment with trypsin, suggesting that integrity of trypsin sensitive sites on NK cell membranes is important for expression of cytotoxicity[88]. The susceptibility of NK cell cytotoxicity to protease activity could be of importance to the escape of tumour cells from cell-mediated destruction (Section 1.5).

In summary, many patients with malignant tumours exhibit specific antitumour immune responses. <u>In vitro</u> experiments show clearly that lymphoid cells (lymphocytes, macrophages and NK cells) are able to destroy tumour cell targets, but only rarely is there <u>in vivo</u> demonstration of successful immune attacks upon primary tumours. The relevance of such observations to a patient's ability to resist the spread and progression of the malignant process is difficult to assess. The dilemma is that although immunological responses directed specifically against malignant cells may occur, they are ineffective in destroying clinically detectable neoplasms

and cannot halt the proliferation of tumour cells. Clearly, there exists a paradox of tumour growth in the presence of antitumour responses. It is as if established tumours have evaded the immune system, a process which is fundamental to the success of transformed cells (Section 1.1.1). Possible mechanisms by which tumours evade the immune system are now reviewed.

1.5 MECHANISMS BY WHICH TUMOURS EVADE IMMUNE DESTRUCTION

Several mechanisms have been proposed to account for the failure of individuals to eliminate their tumours. Many reports have described generalised immunological impairment in untreated cancer patients, although immune defects are not found uniformly in all cancer patients[94]. There is some experimental evidence to suggest that the presence of malignant tissue per se may lead to host immune depression. In mice, for example, the number of antibody-forming cells was found to decrease gradually with increasing numbers of Ehrlich ascites tumour cells, the impaired response being seen as a reduction in antibody production rather than a delay in the timing of the response[45]. Other studies failed to detect generalised depression of immune cells in peripheral blood but found functional impairment of host cells which had infiltrated carcinomas of the colon[48] and lung[71]. Recently, Holmes[96] attempted to define the functional capacity of tumour-infiltrating lymphocytes and PBL in patients undergoing pulmonary resection for primary adenocarcinoma or squamous cell carcinoma, or lung metastases from carcinomas of the kidney or colon, malignant melanoma or soft tissue sarcomas; using mixed lymphocyte culture (MLC) and chromium release cytotoxicity assays, Holmes found that the functional capacity of tumour-infiltrating lymphocytes and NK cells was suppressed dramatically in comparison with PBL and NK cells, although the proportions of T and B lymphocytes, Ts and Th cells and NK cells were similar within

the tumour and peripheral blood. These studies[96] suggest strongly that the tumour micro-environment has a profound suppressive influence on host immune cells and that this immunosuppressive micro-environment serves as a protective mechanism against host cells.

There are several mechanisms by which local and systemic immune cells could be suppressed by tumours.

1.5.1 GENETIC CONTROL

Genetic control of immune responses to complex antigens has been reviewed in Section 1.3.1. Animal studies support the concept that immune reactions to tumours are under the control of Ir genes. Lilly[97] found that susceptibility of mice to the Gross leukaemia virus is controlled by two genes, one of which is linked to the H-2 system: mice without this gene are about ten times more susceptible than those with the "resistance gene". Similarly, Kolsch[98] showed that low responsiveness of DBA/1 mice to an allogeneic (DBA/2) mastocytoma P-815 ($H\text{-}2^d$) was inherited in a recessive pattern and was due to a determinant-specific defect in T cell mediated cytotoxicity and an inability of the DBA/1 host to produce a secondary humoral anti-H 2^d response.

The MHC has a role in determining the ability of a host to respond to some complex antigens (Section 1.4.1). Also, the ability, or otherwise, to express H-2 gene products at the tumour cell surface has been shown to be an important determinant of the murine immune response to chemically-induced tumours[46]. The importance of Ir genes in the susceptibility of humans to cancer or their ability to overcome malignant disease is largely unknown. However, the high incidence of cancer in certain families[99] and in some pre-existing medical conditions (e.g. carcinoma of the colon with

polyposis coli), and the ability of some individuals to induce enzymes to detoxify chemical carcinogens[100] indicate that genetic factors do have some influence on the occurrence of malignancy, and may influence the host's ability to respond to the presence of malignant tissue.

1.5.2 IMMUNOLOGICALLY INACCESSIBLE SITES

Some parts of the body are said to be "immunologically privileged" in that they are protected from mediators of immune reactions - for example, the anterior eye chamber and central nervous system. Tumours are rare in such sites, probably because there are relatively few cells which have very low mitotic rates - features which are not associated with tumourogenesis.

Tumours in the eye and brain are not associated usually with extensive inflammatory infiltrates. However, Cochran[101] has detected evidence of CMI and humoral responses against ocular melanomas at almost the same frequency as cutaneous melanomas, although of course ocular melanomas do not arise within the anterior eye chamber. Overall, it is very unlikely that immunological privilege within anatomical sites plays a significant part in evasion by tumours in humans.

1.5.3 CHANGES AT THE TUMOUR CELL MEMBRANE

Cell membranes are dynamic structures and continually undergo degradation and synthesis of constituent proteins, glycoproteins and lipids. Thus, replacement of antigenic membrane components without loss of cell viability but with less antigenic molecular configurations represents a mechanism by which tumour cells could escape detection or destruction by the immune system.

1.5.3 (a) Antigenic Modulation

Antigenic modulation has been demonstrated by Ioachim[102-105] in rat thymomas induced by the Gross murine leukaemia virus (GMuLV) and maintained continuously in tissue culture, where they strongly express specific viral surface antigens. Tumour cells which expressed the viral antigen (i.e. GMuLV +ve cells) were rejected consistently and were unable to produce tumours in normal adult syngeneic rats but grew progressively in irradiated immunodeficient rats. After passage into irradiated adults or immature infant rats, the GMuLV +ve cells lost their GMuLV surface antigen; these GMuLV -ve tumour cells had greatly increased malignant potential in that they grew progressively in normal immunocompetent syngeneic adult rats and metastasised widely. Thus, malignant potential was related to expression of surface antigens which could be modified by an ineffective immune response; after in vivo antigenic conversion, the enhanced malignant potential was passed on and persisted in subsequent generations of tumour cells.

These experimental studies show that when the immune response did not destroy tumour cells, substantial alterations in antigenic expression and immunogenicity were induced. Therefore, an inadequate antibody response can induce antigenic change which not only allows tumour cells to escape immune destruction but also endows them with greater malignant potential; highly immunogenic cells may be destroyed but cells with poor antigenicity can be induced, thereby ensuring that the most malignant tumour cells are cloned. This mechanism could explain in part the association of a local antibody response to tumours with an unfavourable prognosis (Section 1.4.2).

Antigenic modulation may be important in the development of metastases. Goldman et al[106] observed that B16 melanoma cells which

formed pulmonary metastases in C57BL/6 mice had reduced antigenicity compared with cells from the subcutaneous primary tumour. Also, antigenicity may be stage-related in some tumours: Black et al[107] demonstrated by skin window tests and leukocyte migration inhibition tests that tumour specific antigenicity was maximal in in situ breast carcinomas and was expressed least in invasive carcinomas with distant metastases, although it is not clear from this study of human breast cancers whether or not antigenic modulation had occurred.

1.5.3 (b) Antigen shedding

Oncofoetal (OFA) and tumour associated antigens (TSA) can be detected in the serum of some individuals with malignant disease. These antigens are tumour products and are released from the surface of tumour cells by the process of "shedding"[108], a mechanism which may help tumour cells escape immunological destruction in several ways.

Firstly, antigen shedding may prevent or interfere with tumouricidal immune responses. Local stability of the target cell antigen and effector cell or immunoglobulin is required for lysis of tumour cells[10]. Antigen shedding removes this stability as well as creating locally high concentrations of antigen which could mop up specific antibody and form immune complexes. Secondly, OFA and TSA may induce host immunosuppression without forming antigen-antibody complexes. For example, alpha-fetoprotein (AFP) is an OFA and has well recognised immunosuppressive properties[109,110], and other tumour antigens have also been shown to suppress immunological reactions[95,126-128].

Shedding of tumour cell surface products is related to the malignant potential of a tumour. Black[108] described two cell lines

derived from a murine mammary adenocarcinoma and grown intraperitoneally as ascites-forming tumours; one cell line, which lost strain specificity so that it could grow in an allogeneic host, was relatively non-immunogenic and had on its surface a large molecular weight glycoprotein which was continually shed into the ascites fluid, while the other cell line grew only in syngeneic animals, was more immunogenic and did not have the surface glycoprotein. Shedding of the glycoprotein was an important determinant of tumour immunogenicity; the glycoprotein may have represented surface antigens or may have masked surface TSA or TATA from immune detection.

1.5.3 (c) Release of proteases

In addition to antigens, a variety of other molecules and membrane structures are shed from the surface of normal and malignant cells[40]. These molecules are associated with cell membranes before being released and so they are said to be "shed" rather than "secreted".

Several protease enzymes are associated with tumour cells. Koono et al[111] isolated four protease enzymes from rat ascites hepatoma cells; following intradermal injection of one of the enzymes (a neutral protease), a factor chemotactic for tumour cells was detected and was able to induce extravascular migration of circulating tumour cells. Sylven et al[112] demonstrated that interstitial fluid from murine tumours had increased proteolytic activity and demonstrated the presence of a variety of enzymes including acid proteases (cathepsin B and D), polypeptidases, dipeptidases, amino peptidases and carboxypeptidases. They suggested that the ability of tumour cells to invade could be due to the activity of these enzymes[112].

Wu[113] demonstrated that glycolytic enzymes as well as pentose phosphate cycle enzymes were released from intact Ehrlich ascites tumour

cells but was uncertain whether these were intracellular or membrane-associated enzymes. Holmberg[114] showed that cytoplasmic enzymes were released from intact Ehrlich ascites tumour cells and that the degree of release correlated with the capacity for successful tumour transplantation into allogeneic mice. Holmberg postulated that "the released cytoplasmic enzymes might be operative in preparing the soil for tumour take"[114].

Several studies have reported a relationship between cell surface protease activity and cell growth rate, with cells with the shortest doubling time having the highest levels of surface protease activity[108]. The presence and release of proteases at the surface of malignant cells suggests that proteolysis is an important feature of malignancy. It has been suggested that tumour-associated proteolysis may be important for the local[112-114] and systemic[111] resistance of tumours to immune destruction. Yefenof et al[115] found that Epstein-Barr virus (EBV)-converted B lymphoma cells had a reduced capacity to shed surface-bound antibodies (compared with EBV negative cells) but had an increased ability to degrade surface antibodies, which, it was proposed, was due to longer exposure of the attached antibody to membrane-associated enzymes.

Other functions of surface proteases have been proposed. Burger[116] showed that cultured mouse fibroblasts escaped contact inhibition and continued to divide following addition of very small amounts of trypsin (0.007% of culture medium). This suggests that the proteolytic enzyme was able to convert the cell surface to the type characteristic of malignant cells as the fibroblasts responded in a manner which, in some respects, resembled cell division exhibited by malignant cells in tissue culture.

Thus, release of proteases may facilitate directly the growth and

spread of tumour cells and may help to protect them against attack by antibody. Somewhat paradoxically, there is good evidence that proteolytic enzymes stimulate the immune system. Trypsin is a potent stimulator of B lymphocytes as measured by lymphocyte transformation[117] and the mitogenic effect of trypsin can be abolished by addition of soybean trypsin inhibitor (SBTI). Lymph node and spleen T cells of hampsters can be stimulated to incorporate ^{3}H-thymidine *in vitro* under serum-free conditions by the proteases trypsin and chymotrypsin[118].

Gisler et al[119] showed that trypsin in small amounts (1-12 μg/ml) in mouse spleen cell cultures potentiated antibody synthesis, and they were able to substitute trypsin for T cells in antibody responses of mice to T-dependent antigens. Although the mechanism of action was not known, the "T substitution" effect of protease was not due simply to transformation of a T-dependent-immunogen to a T-independent-immunogen[119]. One possible mode of action of the neutral protease would be by enzymatic removal of "natural blocking factors" from the lymphocyte membrane[120]. Indeed, trypsin can induce structural changes in the glycocalyx (cell coat) of lymphocytes[108] and other proteases can alter the electrophoretic mobility and migration of lymphocytes[121], and it is possible that proteases could act directly on other activities of immune cells. For example, protease enzymes inhibit macrophage migration, in the same manner as MIF[122].

The data reviewed above indicate that proteases or proteolytic events have a role in the activation of T and B lymphocytes. Similarly, protease inhibitors have immunoregulatory activity by their inhibitory effect on protease activity. Hart et al[118] showed that the stimulatory effect of trypsin and chymotrypsin on B lymphocytes was abolished by SBTI. Other studies have shown that protease inhibitors can suppress lymphocyte responses *in vitro*[123].

Cells other than lymphocytes and macrophages are influenced by protease enzymes. Chen et al[124] have shown that the protease thrombin is able to stimulate DNA synthesis and cell reproduction in resting chick embryo fibroblasts *in vitro* and suggested that this action was due to cleavage of growth-regulating proteins. Chen et al[125] also suggested that proteases played a part in host defence at sites of tissue injury and that thrombin and other proteases could act as non-specific B cell stimulants during wound healing. An attractive hypothesis concerning the activities of protease inhibitors can be proposed by developing these suggestions further. Within the area of a wound, a relatively high concentration of active thrombin could stimulate B cells non-specifically as an adjunct to specific stimulation by foreign antigens (e.g. bacteria) and could also act as a stimulus to wound healing by an effect on fibroblasts, in addition to acting on specific substrate (fibrinogen). Other proteases released from damaged tissues would stimulate lymphocytes with subsequent development of an inflammatory response. One component in the regulation of such a response would be the naturally occurring plasma protease inhibitors.

This hypothesis is suggested largely by results of *in vitro* experiments but nevertheless it may be relevant to some aspects of the biological behaviour of tumours. The amount of protease enzymes in a wound is finite so long as the wounding stimulus (or any stimuli of continuing tissue death) has been removed; in the case of a tumour, there would be a constant source of protease enzymes shed from cell surfaces. The ever-increasing release of proteases must benefit the tumour by aiding local invasion, encouraging release of cells from the primary tumour mass, deregulating cellular growth and releasing surface immunoglobulins. On the other hand, proteases could adversely affect tumour cell growth by

encouraging an antibody response, inhibiting macrophage migration and perhaps encouraging a fibroblastic reaction. As the value to the host of the local antibody response is dubious (Sections 1.4.2 and 1.5.5), the potentially harmful effects of tumour proteases would probably outweigh the beneficial effects, in theory at least.

In summary, tumour-related or -associated proteases have wide-ranging effects on the function of the host's immune system and the behaviour of tumour cells. It is not surprising therefore that a complex system of naturally occurring protease inhibitors exists in humans. The role of protease inhibitors in manipulation of the immune response is reviewed in Section 1.5.8.

1.5.4 SUPPRESSOR T LYMPHOCYTES (Ts)

Stimulation by foreign antigens induces both antigen specific and non-specific Ts which have a central role in regulating or restricting immune responses and inducing antigenic tolerance (Section 1.3.2). Thus, Ts might be involved in suppressive mechanisms associated with malignant tumours, and experimental evidence supports this view.

Treves et al[129] demonstrated that spleen cells from C57BL/6 mice bearing Lewis lung carcinomas enhanced tumour growth when injected together with tumour cells into syngeneic animals; removal of T lymphocytes by treatment with anti-Thy serum reduced the enhancing effect and thymectomy before tumour transplantation led to a significant reduction in the number of tumour metastases. Direct evidence of T cell-dependent suppression with specificity for tumour-associated antigens was provided by Fisher et al[130] who demonstrated a T cell population with suppressive activity and specificity for syngeneic, but not allogeneic, ultraviolet- and chemically-

induced tumours. Studies by Greene and colleagues[131-133], showed that "immunosuppressor" T cells were generated in A/Jax mice within 24 hours of transplantation of a methylcholanthrene-induced sarcoma and disappeared within five days of tumour excision.

The situation in humans is less clear, partly because of the need to rely on in vitro assay systems and the large number of variables which influence human immune functions. However, Ts have been shown to contribute to the generalised reduction in immune competence observed in many patients with malignant disease[134]. Yu et al[135] reported the presence of specific suppressor cells which inhibited the cytotoxic activity of autologous tumour-specific cytotoxic lymphocytes in some of their patients with osteogenic sarcoma; PBL contained two subpopulations, one exhibiting tumour-specific cytotoxicity and the second inhibiting this activity. The existence of Ts is likely to be secondary to the tumour as the immune deficit in many patients tends to parallel the extent of disease. The Ts found in patients with lymphoma probably play a more direct role in the pathogenesis of an associated immune defect which is frequently evident early in the course of the disease.

1.5.5 "ANTI-TUMOUR" ANTIBODIES

The obvious function of antibodies produced in response to tumour antigens is tumour cell destruction, and such has been demonstrated in some human and experimental animal systems (Section 1.4.2). However, anti-tumour antibodies are not successful universally, and some evidence suggests that the antibody response may affect the host adversely.

The presence of plasma cells within human malignant melanomas has been associated with a poor prognosis while lymphocytic infiltration has been correlated with a good prognosis[69]. Izsak et al[136] prospectively

studied 25 patients with malignant disease of the breast, gastrointestinal or genito-urinary tracts, or soft tissue sarcomas, and found a close correlation between the presence of tumour cell surface immunoglobulins and a "malignancy index" which was calculated by scoring parameters such as sites of spread, histopathological grade, rate of tumour growth, and an "ability index method" which expressed "somatic objective and subjective criteria"; nine of 12 highly malignant tumours, but only 5 of 13 tumours with a low malignancy rating, were associated with surface immunoglobulin. The study suffers because of the heterogeneous nature of the tumours and the uncertain validity of some of the methods used to assess prognosis, but nevertheless a correlation was found between malignancy and surface antibody. The authors could not explain the presence of antibody on "low malignancy" tumours, but this could have been due to non-specific absorption.

Antibodies may impair the immune response against malignant cells by several mechanisms. Firstly, formation of anti-idiotypic antibodies can suppress generation of cell-mediated and antibody responses (Section 1.3.3). Secondly, specific antibodies can bind to tumour cell surface antigens and prevent them from stimulating further immune responses. Ting et al[137] demonstrated that pre-coating of tumour cells with syngeneic tumour-specific antibodies reduced the capacity of cells to provoke <u>in vivo</u> anti-tumour immunity, and Vanky et al[138] showed an inverse relationship between the presence of immunoglobulins in tumour biopsies and the capacity to stimulate autologous lymphocytes as measured by increased DNA synthesis.

Thirdly, antibodies may facilitate the escape of tumour cells from immune detection and destruction. Antigenic modulation can be seen as a

mechanism of tumour evasion (Section 1.5.3(a)) and has been observed following combination of specific antibody with surface determinants of tumour cells[139]. Witz[140] noted that release of amino acids from tumour cells incubated with specific antibody was increased significantly, and interpreted this as evidence of "enhanced membrane catabolism" which enabled tumours to respond to surface binding of immunoglobulins by increased shedding of surface proteins.

Some tumour cells are able to rid themselves of surface antibody by "antibody uncoating"[141]. Fish et al[142] showed that most of the IgG released from murine ascites TA3/SK mammary carcinoma cells was in a degraded form, indicating that the specific antibody had been "processed" by the tumour, possibly by surface protease enzymes; not only were these degraded cytotoxic antibody products inactive, but they were able to block the cytotoxic capacity of intact antibody because of much higher affinity for tumour cells. Presumably, immunoglobulin degradation could account for the apparent absence of anti-tumour antibodies in the circulation of some patients with malignant tumours (Section 1.4.2).

In summary, antibody responses against tumours are not always favourable to the host and some tumours can alter specific antibodies by surface activities which result not only in loss of the anti-tumour humoral response but also in the appearance of products which are detrimental to the host response. In this way, the humoral response against tumours may aid, paradoxically, tumour cell escape from immune destruction.

1.5.6 COMPLEMENT

Although complement dependent lysis (CDL) is a potential means of eliminating tumour cells (Section 1.4.2), it is not universally successful *in vivo*. The presence of complement components in malignant tissue *per se*

does not indicate necessarily that complement has been taken up by antibody-coated tumour cells: complement may be taken up by host cells with complement receptors, thereby reducing its availability for CDL, which is ineffective in certain phases of the cell cycle in some tumour lines[143]. Also, neoplastic cells differ greatly in their susceptibility to CDL and complement inactivating enzymes have been demonstrated on the surface of some tumour cells[144].

A full review of the role of complement in malignant disease is beyond the scope of this thesis but it is clear that some tumours are able to resist the action of tumour-specific antibody and complement.

1.5.7 CIRCULATING NON-SPECIFIC IMMUNOSUPPRESSIVE FACTORS

Several recent studies indicate that circulating non-specific immunosuppressive factors (CN-SIF) are present in patients with malignant disease. Surprisingly, very few studies have examined more than a single factor and so it has been impossible to assess the relative contributions of such factors to the immunological impairment observed in many cancer patients.

1.5.7 (a) Immune complexes

Immune complexes (IC) in cancer patients are formed by combination of cell surface antigens with antibody, either within the circulation after antigen shedding[145] (Section 1.5.3(b)) or at the tumour cell surface before shedding occurs [141]. IC are detected in about 80% of cancer sera[145].

The presence of IC provides further evidence that antigen shedding from tumour cells represents a method of immune evasion. Using a

methylcholanthrene-induced fibrosarcoma rat model, Thomson et al[146] found that specific antibody could only just be detected when tumours were present but that antibody levels increased significantly within days of tumour excision; injection of tumour cell suspensions into immunised animals resulted in a fall of antibody levels, indicating that constant release of tumour antigens was able to neutralise circulating antibody by the formation of IC[147,148]. The IC in ascitic and pleural fluid secondary to disseminated carcinoma of the breast and ovary have been shown to block in vitro lymphocyte-mediated cytotoxicity[149].

IC affect host resistance adversely by other means also. The Fc portions of antibody molecules in IC can attach to Fc receptors of lymphoid effector cells such as cytotoxic T cells, macrophages and killer cells; as the Fab part of the antibody is attached to free tumour antigen, the effector cell is prevented from attaching to tumour cells, and so this means of immune attack is lost.

1.5.7 (b) Fc fragments

The protease enzyme papain splits the IgG molecule into three fragments: two antigen binding fragments (Fab) which are responsible for antibody specificity, and a fragment which can cystallize (Fc fragment). The Fc fragment cannot combine with antigen but is responsible for the capacity of intact immunoglobulin molecules to activate complement and to bind to cells with Fc receptors, such as most B lymphocytes, monocytes, killer cells, some T cells (especially when activated) and platelets.

Fc receptors are present on some, but not all, cells of human malignant tumours (tumours of lung, stomach, colon, breast, bladder, ovary and thyroid[150]). Even Fc receptor-positive cells express them to varying degrees. Fc receptors on tumour cells are indistinguishable antigenically

from those of immune cells. The function of Fc receptors on tumour cells is not known. Presumably, if immunoglobulin molecules combined with tumour cells at their Fc part rather than at the antigen specific Fab part, the Fc region would be unavailable for activation of complement or attachment of immune effector cells. Also, unrelated antibody could attach to tumour cells by way of Fc components and cause steric interference with binding of specific antibody.

Tumour cell-surface protease enzymes cleave specific antibody molecules so that the Fc portion is released and the Fab portion remains attached to the antigenic determinant on the cell surface. Thus, antigenic determinant sites remain covered by Fab fragments and therefore shielded from immune detection and attack, and antibody-dependent mechanisms of tumour destruction, such as ADCC and CDL (Section 1.4.2), cease to be effective because they require intact antibody to endow effector cells with specificity (ADCC) or to activate complement (CDL).

Fc fragments released from the tumour into the surrounding environment find their way into the circulation. The effect of circulating Fc fragments on immune function is uncertain. However, as many immune cells have Fc surface receptors, free Fc fragments could combine with these receptors and interfere with immune activities. Studies[151] of inactivation of antigen-sensitive B lymphocytes by IC indicate that inactivation is mediated by a direct Fc-dependent effect on lymphocytes and that the Fc portion of immunoglobulin molecules and Fc receptors on immune cells are responsible for some forms of immune regulation. As the Fc subunit is not antigen specific, inactivation by Fc fragments is non-specific; antigen specific forms of antibody feedback could be achieved by specific attachment of Fab fragments to antigenic determinants[151]. The

detection of Fc fragments and their possible effect on immune reactivity in patients with malignant disease is reported in Chapter 3.

1.5.7 (c) Prostaglandins

Prostaglandins (PG) have been implicated in the regulation of humoral and cellular immune responses and in the depressed immune function which is associated frequently with malignant disease. A detailed review of the role of PG in immunological functions is beyond the scope of this work but mention should be made of the possibility that PG could aid tumours in immunological evasion.

PG have a number of inhibitory effects on immune cells including suppression of mitogen-induced transformation of human PBL[152], inhibition of lymphokine production by stimulated lymphocytes[153], suppression of NK cell activity[154] and activation of B lymphocytes[155]. As some lymphokines (e.g. MIF) stimulate PG synthesis by macrophages, it has been suggested that PG act by negative feedback in the control of CMI[153].

PG are produced by some tumours in experimental animals[156] and by some human malignant tumours, such as anaplastic and medullary carcinomas of the thyroid[157], carcinoma of the oesophagus[157] and Kaposi's sarcoma[158]. Indirect evidence of PG-mediate immune depression has come from observations that PG synthetase enzyme inhibitors, such as Indomethacin and Aspirin, can reverse tumour-induced immunosuppression in vivo and in vitro and retard tumour growth in vivo[159]. Other reports[160] have claimed that PG-producing suppressor cells are responsible in part for the associated impairment of in vitro reactivity of PBL in patients with Hodgkin's disease, as suppression can be ameliorated by Indomethacin.

1.5.7 (d) Small molecular weight substances

In addition to the blocking activities of antibodies, tumour antigens and immune complexes (reviewed by Smith and Landy[161]), substances of small molecular weight (MW) and possessing immunosuppressive activity have been detected in sera from cancer patients. The precise nature of such substances is obscure but several reports suggest that they are polypeptides.

Holmberg[162] characterised a peptide obtained by dialysis of cell-free ascitic fluid from humans with advanced malignancies, and found that the peptide (MW 1900-2000) decreased mitotic rates of malignant and non-malignant cell populations. Glasgow et al[163] found that the presence of "immunosuppressive serum" (as determined by inhibition of PHA-induced in vitro lymphocyte transformation) was associated strongly with absence of CDHR to PPD, streptokinase-streptodornase and mumps skin test antigen in a large number of patients with solid malignant tumours; analysis of the sera by cellulose ion-exchange chromatography revealed a peptide of MW less than 10,000; it was derived from the alpha globulin region and caused marked inhibition of in vitro lymphocyte transformation and antibody responses of mice.

Friedman et al[164] showed that inhibition of murine antibody responses induced by tissue cultured mastocytomas was due to a soluble factor of MW less than 5000 daltons; ascitic fluid from mastocytoma-bearing mice contained an additional immunosuppressive fraction of MW 10,000-20,000 daltons. Friedman[164] interpreted these results as indicating that the mastocytoma tumour cells secreted a small MW immunosuppressive material. Unfortunately, the suppressive substances were not characterised, although the paper states that an antibody raised in the rabbit against the low MW substance could reverse its immunosuppressive activity.

The origin of CN-SIF of small MW is uncertain. They could represent whole molecules or fragments of larger molecules shed from the surface of tumour cells, or they could be products of proteins or antibodies degraded by tumour-associated protease enzymes. They could also be products of the host's immune cells.

There is now a considerable literature concerning soluble factors released from immune cells as mediators of immune responses. Such factors have either a helper or suppressor action and normally a balance exists between the two. Soluble mediators are classified as either non-antigen-specific (i.e., they do not bear cell surface-related antigenic structures) or antigen specific (i.e. they bind antigen in order to function). It has been proposed[165] that non-antigen-specific factors are released from immune cells only during direct cell-to-cell interaction - widespread production and diffusion of these mediators during an immune response would be dangerous to the host, as both helper and suppressor factors may feed back on distant organs and have effects on precursor cells[166]. Bankhurst[167] recently reviewed the subject of soluble suppressive factors released by suppressor cells in patients with malignant disease. Several <u>in vitro</u> studies[168-169] have indicated that non-specific soluble suppressor factors are released from normal T cells in cancer-bearing hosts and it is likely that such factors are important in intercellular communication and regulation of immune responses. The relevance of these factors to the immunosuppressive activity of plasma in cancer patients is unknown. Presumably, soluble mediators affect the local immune response to malignant cells and influence activities of immune cells in and around tumours such that immune reactivity is regulated.

Immunosuppressive small MW substances are found circulating in

patients with diseases other than cancer. So called "middle molecules" with a MW range of 300-5000 daltons accumulate in the plasma of patients with chronic renal failure and are able to suppress lymphocyte transformation[170] and mixed lymphocyte reactions[171], significantly prolong skin allograft survival and inhibit graft versus host reactions in rodents[171]. Recently, Mabuchi et al[172] characterised these peptides by high performance liquid chromatography and concluded that these "middle molecules" were normal body constituents present in uraemic blood in abnormally high concentrations because of impaired excretion or metabolic degradation by diseased kidneys. It is difficult to know if these CN-SIF bear any relationship to the small MW suppressive molecules described in patients and experimental animals with tumours. Of course, another explanation for high circulating levels of such substances is excessive production: the membrane of the cancer cell is a very active organelle and if these small MW CN-SIF are tumour products, large quantities would be produced by metabolically active tumours.

In summary, small MW substances with the ability to suppress immune functions have been detected in sera from cancer patients and although their biological significance and origin is uncertain, it is conceivable that they are tumour products which help the tumour to evade immune destruction.

1.5.7 (e) Acute phase reactant proteins

Acute phase reactant proteins (APRP) are largely glycoproteins which alter their concentration in plasma in response to tissue injury, acute and chronic inflammation, connective tissue disorders and some malignant diseases. Interest in APRP with respect to cancer has been from the viewpoints of (i) diagnosis and monitoring, and (ii) the biological

significance and consequences of changes in APRP. Because of poor sensitivity and specificity for cancer, APRP currently have little part in screening and monitoring programmes of patients suspected of or having had malignant disease.

The subject of APRP has been reviewed extensively[173-175]. The APRP of relevance to human malignant diseases are shown in Table 1.1. Elevated plasma levels of some or all of these APRP have been reported in patients with carcinomas of the breast, prostate, stomach, colon, lung, bladder and liver as well as lymphomas and leukaemia[175]. In general, a rough correlation exists between progressive increases in some APRP concentrations and continued tumour growth; cytoreductive therapy may be associated with return of APRP levels to the normal range of values, and recurrence and further tumour growth may be associated with abnormally high levels. The biological activity of some APRP (e.g. caeruloplasmin, fibrinogen) is well defined although the reasons for increased serum levels in some malignant conditions is uncertain.

Some APRP influence the activities of immune cells. C-reactive protein binds selectively to T lymphocytes and inhibits their ability to form spontaneous rosettes with sheep red blood cells and their responses to allogeneic cells in MLC[176]. Alpha-1-acid glycoprotein inhibits proliferative responses of PBL to PHA, Con A and allogeneic cells in MLC[177]. The alpha globulin group of APRP inhibits an extensive range of T cell-mediated immune responses, such as skin allograft rejection, elaboration of MIF, and lymphocyte blastogenesis induced by MLC and PHA stimulation[178].

The relevance of these largely <u>in vitro</u> findings to <u>in vivo</u> biological systems is uncertain. However, demonstration that some APRP are

Table 1.1 Acute phase reactant proteins relevant to human malignant disease

PROTEIN	MW ($x10^{-3}$)	NORMAL RANGE (serum) (g/l)
Alpha-1-acid glycoprotein	40	0.55 - 1.4
Alpha-1-antitrypsin	54	2.0 - 4.0
Alpha-1-antichymotrypsin	68	0.3 - 0.6
Caeruloplasmin	51	0.15 - 0.6
C-reactive protein		<10 mg/l
Haptoglobin type 1-1	100	1.0 - 2.2
type 2-1	200	1.6 - 3.0
type 2-2	400	1.2 - 2.6
Fibrinogen	340	2.0 - 4.5
Alpha-2-macroglobulin	725	1.0 - 3.0

increased in cancer patients (alpha-2-macroglobulin being a notable exception) and have immunosuppressive properties leads to the conclusion that APRP could be partly responsible for the immunodepression associated with malignancy and could facilitate growth and progression of tumours.

1.5.8 ALPHA - 2 - MACROGLOBULIN

The alpha globulins of human plasma may be separated electrophoretically into alpha-1-globulins (glycoproteins and high density lipoproteins) and alpha-2-globulins (alpha-2-macroglobulin (A2M), haptoglobin, caeruloplasmin, prothrombin, very low density lipoproteins and some glycoproteins). From a functional viewpoint, about 20% of the globulin fraction of plasma consists of proteolytic enzyme inhibitors of which the heavy MW alpha-2-globulin, A2M, is a major component. The literature concerning A2M is extensive, and the biological and chemical characteristics of the molecule have been reviewed recently[179-185]. The following review will outline briefly the biochemical and physiological properties of A2M and the relevance of this molecule to human disease.

A2M (MW 725,000 daltons) is composed of two non-covalently bonded pairs of identical subunits joined by disulphide bonds such that the molecule has a generally cylindrical shape[186]. Each of the four subunits contains a specific amino acid sequence (the "bait" region) which is highly susceptible to proteolytic cleavage, following which the whole A2M molecule undergoes a conformational change; this results in irreversible binding of the protease enzyme without blocking the enzyme's active proteolytic site[187]. Small MW substances (e.g. aprotinin, MW 6511) are able to gain access to the active centre of a protease enzyme complexed with A2M, whereas inhibitors with MW greater than 9000 (e.g. SBTI) cease to have any influence on the enzyme.

Electron microscopy[184,187] and gel electrophoresis studies[184] reveal that the A2M-enzyme complex is conformationally and electrophoretically distinct from free or non-complexed native A2M, the electrophoretically fast nature of complexed A2M being due to the molecule's more compact configuration.

Human A2M is unique among the numerous naturally occurring plasma protease inhibitors in its ability to inhibit all four classes of endopeptidases with nearly any specificity[183] (Table 1.2). After binding with enzymes, a receptor-recognition site on the A2M molecule is exposed so that the complex is cleared rapidly and specifically from the circulation by hepatocytes, fibroblasts and reticuloendothelial cells in the liver, spleen and bone marrow[188,189]. The daily fractional turnover of free circulating A2M is approximately 10%, (half life = 9-10 days) whereas A2M-protease (A2M-P) complexes have a half life of between 4 and 8 minutes[188,190]. The rapid elimination of A2M-P suggests that the biological importance of A2M is in removing proteases from the circulation[190].

The site of synthesis of A2M is uncertain but hepatocytes, monocytes and lymphocytes in tissue culture can synthesise A2M[182,191]. A2M is distributed widely in body fluids (plasma, extracellular fluid, synovial fluid, pleural and peritoneal effusions, seminal fluid), and A2M is a surface component of human and murine B lymphocytes[192] and human platelets[181]. Unlike other plasma protease inhibitors, A2M forms a more or less continuous lining on the luminal surface of human vascular endothelium[193].

Several studies have reported A2M concentrations in normal subjects and patients with various diseases. Generally, A2M levels in serum are

Table 1.2 Substances which bind with alpha-2-macroglobulin (from James[180])

Serine proteases	-	Trypsin, chymotrypsin, plasmin, thrombin, kallikrein, plasminogen activator, urokinase, polymorphonuclear leukocyte elastase
Thiol protease	-	Cathepsin B1
Carboxyl proteases	-	Cathepsin D, some acid proteases
Metal proteases	-	Collagenases, thermolysin
Ions	-	Nickel, zinc, lithium
Lymphokines	-	Macrophage activating factor, macrophage slowing factor
Mitogens	-	PHA, Con A
Miscellaneous	-	Methylamine, soluble Ia antigens, immune complexes

higher in females than males, in infants than adults, and may be elevated in some patients with diseases such as diabetes mellitus, atopic dermatitis, asthma, agammaglobulinaemia, prostatic carcinoma and dwarfism secondary to zinc deficiency[194-198]. Concentrations (mean ± ISD) of A2M measured by radial immunodiffusion in sera from healthy subjects were 2.65 ± 0.55 g/l (males, n=33) and 3.35 ± 0.57 g/l (females, n=22)[194]. Absolute levels in plasma or serum provide little information about the functional activity of the molecule. Farrow et al[199] studied metabolic turnover of ^{131}I labelled non-complexed A2M in 4 normal subjects and 7 patients with burns to 9-10% of their body surface, and found that although total intravascular A2M concentration remained relatively normal, A2M turnover increased to twice normal in the burnt subjects. It is well recognised that burnt patients are relatively immunosuppressed and that their serum has immunosuppressive effects in vitro[200], but unfortunately concurrent immunological tests were not performed[199].

The vital biological importance of A2M is suggested by its presence in nearly all mammalian species and by the fact that complete deficiency has not been described in humans, although a family with an inherited relative deficiency of A2M has been reported recently[201].

The precise physiological role of A2M remains obscure. A2M is probably the most important of the major plasma protease inhibitors as it can bind non-specifically and irreversibly to almost any active proteolytic enzyme which is either freely circulating or already bound to other protease inhibitors. After formation of A2M-P complexes, activity of protease enzymes is reduced and the complex is eliminated rapidly by cells with specific receptors for A2M-P but not free A2M. Therefore, it is likely that a major role of A2M is removal of protease enzymes, and thereby limitation of the many physiological and inflammatory events which are

mediated by the action of proteolytic enzymes (e.g. the blood coagulation cascade, the fibrinolytic pathway and complement activation mechanisms).

Interaction of A2M with cells of the immune system is poorly understood but there appears to be a relationship. A2M has been associated with surface membranes of B lymphocytes in humans and mice[202]. Lymphocytes[203] and monocytes[204] synthesise and release A2M. A2M blocks antigen-induced production of lymphokine by obscuring antigen recognition, blockade being greatest when A2M is obtained from pathological sera[205]. (It is of interest to the present study that the authors[205] used an in vitro cellular electrophoretic method of detecting responsiveness of sensitised lymphocytes, a subject reviewed in Section 2.3). Remold et al[25,26] found that A2M enhanced the response of guinea pig macrophages to the action of MIF (Section 1.2.1). McDaniel et al[206] showed that A2M binds lymphokines and suggested that the molecule has a carrier function with respect to small MW mediators of cellular immunity. Streilein et al[207] showed that A2M depressed lymphocyte blastogenesis to B cell mitogens in the hamster.

The biochemical data and functional activities have been brought together by Hubbard[208] to form an hypothesis concerning the physiological role of A2M. Hubbard proposed that the immunosuppressive quality of A2M is due to "denaturative alteration" as a result of protease binding[208], an hypothesis which explains the facts that (i) A2M is apparently immunosuppressive in some circumstances and yet relatively large amounts of A2M circulate in healthy immunocompetent individuals, and (ii) that target cell recognition for the suppressive effect and for clearance appears to be dependent on conformational change of the molecule. Hubbard also speculated that as the A2M-P complex is generated by proteolytic activity

at the A2M "bait" region and that as A2M-P is eliminated and degraded rapidly by macrophages[209], peptide fragments should be generated by cleavage of any of the four A2M subunits following A2M-protease interaction. At this stage, the experimental work described in this thesis was undertaken to investigate the hypotheses proposed in Section 1.8.

1.6 MALNUTRITION AND CANCER

Anorexia and weight loss are common symptoms among cancer patients, many of whom have measurable nutritional abnormalities at diagnosis. The clinical syndrome of cancer malnutrition or "cancer cachexia" is typified by the patient who, in addition to symptoms specifically related to his tumour, has illness-related loss of body fat, muscle wasting, anaemia, generalised weakness, lethargy and an associated rise in basal metabolic rate[210]. The mechanisms responsible for this syndrome are complex but include reduced intake of food, loss of gastrointestinal mobility, malabsorption, mechanical restrictions due to the anatomical location of the tumour, tumour ulceration and bleeding, altered metabolism due to the presence of the tumour, superadded infection or intercurrent illness, and fasting because of investigations and treatment.

The tremendous interest in nutrition and nutritional therapy within the last 10-15 years has been due in part to the realisation that nutritional impairment of patients with benign or malignant disease has serious clinical sequelae. As early as 1936 Studley[211] demonstrated that the mortality of patients undergoing surgery for benign peptic ulcer was ten times greater in those who had lost 20% or more of body weight than in those without weight loss, and in many cases death was attributable to septic complications.

More recently, Simms et al[212] studied post-operative

complications in relation to nutritional status in 97 patients with cancer and 19 with inflammatory bowel disease and found that complications had a direct relationship with poor nutritional status; interestingly, the distribution of complications was related to nutritional status, not the presence of malignancy.

The increase in morbidity and mortality in malnourished patients reflects the wide ranging effects of impaired nutrition on virtually all body tissues. Body composition studies[264,265] have been undertaken recently using total body neutron activation analysis (TBNAA) techniques (Section 1.6.2(c)), to identify alterations in body composition and to determine the relationship between dietary intake and body composition in cancer patients. Body fat and muscle were often reduced significantly in patients with gastrointestinal cancers in comparison with age- and sex matched control subjects: non-muscle lean tissue remained approximately the same but assumed a greater proportion of body weight[264]. In patients experiencing weight loss over a six month period of observation, protein, body water and fat were lost, and their mean intake of energy, protein and fat was reduced markedly[266].

The precise cause for these body composition defects is related to caloric intake and metabolic effects of the tumour. Cohn et al[265] studied prospectively groups of patients with gastrointestinal, lung and head and neck malignancies during periods of chemotherapy and found that the difference between those who gained weight and those who lost weight was related more to their protein and calorie intake than to their tumour burden or the administration of chemotherapy. Similarly, Burke et al[267] found no significant difference in dietary intake or metabolic rate between patients with cancer who maintained their weight and those with benign

disease, but that energy intake was lower in those cancer patients who lost weight. Patients with inadequate calorie intake have to use their non-visceral protein and fat stores as energy supplies.

The influence of an actively growing tumour cannot be disregarded however. In an eloquent study of whole body protein turnover using a radiolabelled leucine infusion technique in patients with colorectal cancer, Carmichael et al[268] found that protein synthesis and breakdown increased with increasing tumour stage but were lower in patients with marked anorexia; anthropometric studies indicated that most patients had lost body protein but, as all were in positive nitrogen balance, the authors concluded that protein stores had been translocated from muscle to sites of more rapid protein synthesis (i.e. their tumours).

Therefore, a tumour places additional requirements for energy and nitrogen on the host and if they are not met by dietary intake, host tissues will be catabolised. Two questions arise with respect to the present study: what are the immunological consequences of the increased catabolism which may be associated with the presence of solid tumours, and, if nutritional therapy is of benefit to cancer patients, does the nature of the nutritional repletion have any bearing on immune status? These two questions will be addressed by the experimental work described in Chapters 3 and 4.

1.6.1 IMMUNOLOGICAL CONSEQUENCES OF MALNUTRITION

The influence of malnutrition on the immune system is difficult to study in malnourished subjects in the western world where malnutrition is nearly always secondary to diseases which themselves may affect immunity. There are several reasons why immunity may be impaired in hospitalised patients with cancer apart from the effects of malnutrition - advanced

age[213], malignancy[214], infection and intercurrent disease[215], and the effects of anaesthesia and surgery[216], blood transfusion[217], radiotherapy[218] and chemotherapy[219] and it may be difficult to implicate one factor more than another. Dominioni et al[220] attempted to evaluate the importance of various factors associated with immune depression in cancer patients by multivariate analysis of impaired CDHR in 111 patients with gastrointestinal and other cancers. Malnutrition and ageing were identified as the two major factors rather than the presence of cancer per se, even if the malignant process was advanced; no significant difference was found in the distribution of anergic, "hypo-ergic" and "normo-ergic" skin responses between cancer patients and 56 patients with benign disorders after correction for nutritional status[220]. This important study perhaps plays down the immunological effects of malignant disease but offers an explanation for the inconsistent findings obtained from skin testing patients with cancer: although CDHR are impaired frequently in cancer patients, this finding is by no means universal[214,220-222].

Brookes et al[223] have provided evidence to support the contention that malnutrition is a potent cause of immunological impairment in cancer patients. In a prospective study of 53 patients with head and neck cancer, Brookes[223] found a 40% incidence of nutritional deficiency which was due largely to local obstruction of the upper gastrointestinal tract by tumour; statistically significant correlations were found between CDHR anergy, lymphopenia and nutritional deficiency as assessed by static anthropometric measurements. Similar relationships were found using different parameters in patients with benign and malignant diseases of the gastrointestinal tract[224]. These studies demonstrate that, in some cancer patients at

least, reduced immunocompetence is secondary to malnutrition rather than the presence of malignancy. Further supportive evidence is provided by studies of nutritional repletion in malnourished cancer patients (Section 1.7.1).

The influence of malnutrition on the immune system has been reviewed extensively[215,221,225,226]. Although not all studies are in complete agreement, it is possible to reach a consensus. Malnutrition leads to adverse effects on specific immunity, with impairment of T lymphocyte activities being most pronounced (Table 1.3). There is relative sparing of B cell function. Impaired antibody responses to T-dependent antigens may reflect reduced Th cell activity rather than B lymphocyte impairment. Neumann et al[227] found that IgE levels were elevated in children suffering from protein-calorie malnutrition when an associated defect in cellular immunity was detectable, although the pathogenesis of this elevation is uncertain. The increased incidence of infective complications in malnourished patients can be explained on the basis of impaired T lymphocyte and phagocytic cell activity and reduced serum levels of complement components, in addition to structural and functional defects in non-specific or innate mechanisms of host resistance.

Apart from studies of peripheral blood concentrations of antibodies, acute phase proteins and nutrients, there are few data about the role of immunoregulatory plasma substances in malnourished subjects. McFarlane[228] reported that subjects with protein-calorie malnutrition had significantly lower plasma levels of alpha-1-antitrypsin, transferrin, C3 and A2M but increased levels of C-reactive protein. Dionigi was able to correlate acute phase reactant protein levels with nutritional status[221]. The significance of these changes is uncertain but, as APRP can influence immune function (Section 1.5.7(e)), changes in their plasma concentrations

Table 1.3 A summary of the effects of malnutrition on the human immune system

(a) Histopathology			
Thymus	- Total weight		Reduced
	- Cellularity		Reduced
	- Hassall's corpuscles		Reduced
Lymph node	- Paracortical areas		Depleted of cells
	- Germinal centres		Reduced in number
Spleen	- Periarteriolar areas		Depleted of cells
	- Primary follicles		Depleted
(b) Peripheral blood lymphocytes			
Total number			Normal or slightly reduced
Proportion of T cells			Reduced
T cell helper : suppressor ratio			Reduced
Null cell number			Slightly increased
B cell number			Often normal
(c) <u>In vivo</u> tests of cell mediated immunity			
Cutaneous delayed hypersensitivity reactions			Consistent reduction in number and size of positive responses
Allograft rejection (animal studies)			Inconsistent results
Graft versus host reaction (animal studies)			Increased reaction
(d) <u>In vitro</u> tests of lymphocyte function			
Lymphocyte transformation in response to:		PHA	Reduced
		PWM	Often reduced
		MLC	Reduced
Th function			Reduced

Table 1.3 (continued)

(d) In vitro tests of lymphocyte function (continued)	
Antibody dependent cellular cytotoxicity	Increased
T lymphocyte mediated cytotoxicity	Increased/decreased
NK cell activity	Increased
(e) Lymphokine production	
Macrophage migration inhibition factor	Reduced
Lymphocyte inhibition factor	Reduced
(f) Polymorphonuclear leukocytes (PML) and macrophages	
Numbers of PML in PB	Normal
Phagocytosis (PML)	Reduced
Random mobility (PML)	Normal
Chemotaxis (PML)	Reduced
Post-phagocytic increase in metabolic activity	Reduced or absent
Microbial capacity	Reduced
(g) Immunoglobulin level in serum	
IgG, IgM, IgA and IgD	Variable but often elevated
IgE	Considerably increased
(h) Antibody responses to antigens	
Thymus dependent antigens	Response depressed
Thymus independent antigens	Response is variable but may be normal
(i) Complement components	
C3, C9 and Factor B	Reduced
C4, C5 and C1 inactivator	Normal

could explain partly the immune changes observed in malnourished individuals. The role of plasma immunoregulatory substances in malnourished individuals is investigated by the experiments described in Section 3.3.

1.6.2 NUTRITIONAL ASSESSMENT

The aims of nutritional assessment of an individual are (a) to identify that the patient is malnourished, (b) to provide an objective and quantitative assessment of the severity of malnutrition, and (c) to allow sequential monitoring of the efficacy of nutritional therapy. Ideally, the method of assessment should be objective, reproducible, straightforward and quickly performed, as well as having a high degree of sensitivity and specificity. The following methods of nutritional assessment have been proposed as suitable for application to hospitalised patients and some have been used in the present work.

1.6.2 (a) Clinical judgement

A wide range of symptoms and signs may suggest that a patient has nutritional impairment, and often there is no dispute among practitioners that a certain patient is malnourished. However, difficulties arise when only a few signs are manifest or when practitioners differ in the interpretation of the patient's clinical features. Similarly, manifestations of the underlying disease may make nutritional assessment difficult by clinical judgement alone. Also, clinical assessment does not provide an objective or quantitative measure of the malnourished state.

A comparative study by Baker et al[229] showed that clinical assessment by two independent practitioners provided a high degree of concordance (81%) and correlated well with anthropometric and biochemical

but not immunological methods of assessment. Although the authors[229] claimed that general clinical assessment was highly reproducible, this is difficult to substantiate without a highly controlled study and perhaps it is significant that attempts at correlating successive assessments following nutritional therapy were not made.

Although helpful in identifying malnourished subjects, clinical assessment was not used as a method of quantitative nutritional assessment in the present study.

1.6.2 (b) Anthropometric measurements

Anthropometric measurements consist of body weight in relation to height, illness-related weight loss, measurement of skin fold thickness and measurement of arm muscle circumference derived from triceps skin fold thickness and mid-arm circumference.

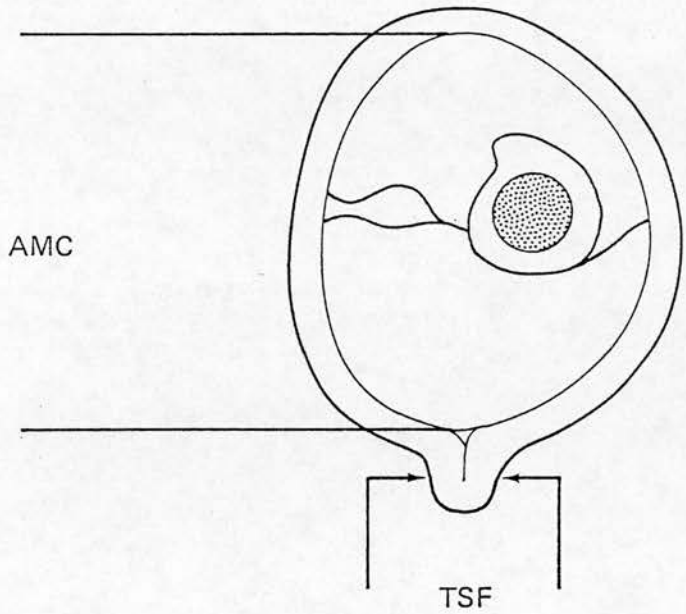
Body weight (BW) provides an overall measure of the total weight of individual body compartments but provides little information about the nature or severity of nutritional impairment, except for extreme values. Changes in body weight reflect changes in any of the labile body compartments, and an increase in one compartment (e.g. extracellular fluid) may obscure a decrease in another (e.g. skeletal muscle) without an accompanying change in BW. As with other anthropometric measurements, BW for a given height is compared with tables of standard BW or "ideal body weight for height" tables[230] to provide an estimate of nutritional status, expressed as a percentage of ideal body weight (IBW). The limitation of this measurement is illustrated by the example of a previously obese patient (120% IBW) who suffers significant illness-related loss of body fat and protein, and at the time of nutritional assessment has

a BW equal to 98% IBW, which is considered relatively normal and which obscures significant nutritional impairment.

Weight loss secondary to illness is a better guide to nutritional impairment and by-passes the need for tables of standard values. Percentage weight loss is calculated from the formula $\frac{UW - PW}{UW} \times 100$ (UW = usual pre-illness weight, PW = present weight). However, this measure is also problematic in that it usually relies upon subject recall for the value of UW, and accurate and regular measurements of weight are recorded only occasionally by the patient or by medical practitioners. Values of weight recalled by memory have been shown to be subject to error[231].

Although weight for height correlates well with body cell mass as determined by body composition radioisotope techniques[232], the value of BW and weight loss parameters in predicting post-operative complications in malnourished surgical patients appears limited. Klidjian et al[233] showed that "BW less than 90% IBW" was correlated significantly ($P<0.05$) with the occurrence of post-operative complications but "weight loss of greater than 10% BW" was not.

Skin fold thickness is the simplest and most widely used measurement of body fat, some 50% of which lies subcutaneously. The skin fold measured most frequently is the triceps skin fold (TSF) which consists of a double layer of skin and subcutaneous fat (Figure 1.1). The method assumes that changes in subcutaneous fat reflect changes in total body fat and that alterations in fat stores occur proportionately throughout the body. The TSF can be measured easily with spring callipers (Section 2.2.4), and repetitive measurements by a single trained observer are highly reproducible[234]. In contrast, measurements made by different observers have poor reproducibility[231].



AMC = Arm muscle circumference
TSF = Triceps skin fold

Figure 1.1 Measurement of triceps skin fold (TSF) thickness at the mid-point of the arm

The value of TSF measurement for detecting malnutrition has been questioned recently, as body fat may remain unchanged or may be excessive in the presence of moderate nutritional impairment. Loss of body fat is probably not as important from a functional point of view as loss of body protein[232]. Measurements of body fat have been shown to be less reliable in detecting malnutrition than estimates of skeletal protein and plasma albumin concentrations[233]. However, the amount of subcutaneous fat indicates the size of energy reserves, and a severe reduction implies reduced ability to withstand prolonged periods of inadequate caloric intake. Mullen et al[235] found that TSF was a predictive parameter of post-operative morbidity and mortality among patients with severe nutritional depletion.

Arm muscle circumference (AMC) is a calculated parameter of lean body mass or skeletal protein mass, and is obtained from the formula AMC = MAC - (TT x TSF), (MAC = mid-arm circumference, TSF = triceps skin fold thickness). Although AMC is used widely in nutritional assessment it has some inherent problems. It assumes that the arm muscles and humerus are circular, which they are not. Secondly, the arm is fusiform in shape in its long axis and so measurement of MAC and TSF must be taken precisely at the same point along the arm each time. Thirdly, the combination of MAC and TSF measurements increases the possible percentage error of the estimation, especially with multiple observers[235].

Opinion is divided as to the value of AMC measurements in nutritional assessment. Mullen et al[236] found that AMC did not predict post-operative complications. Conversely, Klidjian et al[233] found that an AMC of less than 85% of a standard AMC was associated significantly ($P<0.02$) with a high risk of post-operative problems, and considered that AMC should be one of three criteria of protein depletion. Further support

for the use of AMC is provided by Harries et al[237] who found a strong positive correlation (r=0.87, P<0.001) between AMC and weight-for-height measurements (expressed as percentages of standard values) in over 100 patients with inflammatory bowel disease, and observed that the "90% of ideal values" cut off point for weight-for-height, AMC and serum albumin was able to distinguish malnourished from well nourished patients.

Anthropometric measurements have two major limitations. First, there may be considerable observer error, especially when multiple observers perform the assessment sequentially. The "multiple observer error" was eliminated in the present study by the author performing all nutritional assessments. Measurements made by single observers have been shown to be reproducible[234,238]. Second, evaluation of anthropometric data relies on comparison of measured parameters with those of a standard or normal population, and problems arise with both the definition of normal for any particular population and the relevance of the reference population to particular patients. The most widely used reference values for weight in relation to height are the Metropolitan Life Insurance Company tables[230,238], while values used for TSF, MAC and AMC may be determined by individual researchers[230]. The relevance of these factors to the present study is discussed in Chapter 6.

1.6.2 (c) Biochemical measurements

Many biochemical measurements have been used to define malnutrition and to identify malnourished individuals. Body composition measurements can be made from TBNAA and radioisotope dilution studies using a whole body counter and the radioisotopes ^{40}K, ^{22}Na and tritiated ("heavy") water. Body potassium is found predominantly in muscle, and total body potassium has

been used for some time as an index of muscle mass and body cell mass. Total exchangeable sodium is used as a measure of extracellular mass which is lean body mass less body cell mass. Total body water provides an index of the fat-free tissues. The ratio of exchangeable sodium to potassium (Na_e/K_e) will increase in cases of continuing nutritional impairment and catabolism[264].

Body composition measurements are attractive methods of assessing nutritional status because they measure directly different body compartments, they are objective, do not rely on standard or ideal values, provide a quantitative assessment and can be used for short-term assessment of nutritional therapy. Although not without sources of error, body composition measurements are regarded as the "gold standard" of nutritional assessment[232,239]. Unfortunately, the techniques are not used widely in clinical practice because of their high capital costs and the requirement for personnel with specialised technical expertise; they are time consuming and cannot be applied at the bed side. Forse et al[232] compared anthropometric and biochemical methods of assessment with the results of body composition studies in 206 normal and malnourished general surgical patients and a small group of morbidly obese patients awaiting weight-reduction surgery; anthropometric and biochemical parameters correlated well with appropriate body composition parameters but there was a wide scatter of results for each parameter. Methods of measurement with low sensitivity are of limited value for assessment of individual subjects but are useful in epidemiological studies.

The 24-hour urinary excretion of creatinine has been used as a biochemical marker for estimation of body muscle mass, and the ratio of 24 hour urinary creatinine excretion of a patient to that of a "normal" subject of the same sex, height and body build has been proposed as an

index of somatic protein depletion ("creatinine/height ratio")[240]. Creatinine is a breakdown product of creatine which is an energy storage protein of muscle and which breaks down spontaneously at a relatively constant rate. Determination of creatinine/height ratio is fraught with problems: it relies on accurate collection of all urine samples over a 24 hour period, a procedure which is extremely difficult to do precisely, even in well controlled metabolic units[240,241]; renal function must be normal; the index changes with increasing age; and interpretation of the result relies on the use of normal values from a healthy population which must be relevant to the patient being assessed.

Similarly, measurement of urinary excretion of 3-methyl-histidine, an amino acid found in myofibrillar but not sarcoplasmic protein, has been proposed as an index of muscle protein turnover and nutritional status[242,243]. The same criticisms can be made of this biochemical parameter as of urinary creatinine estimation. In addition, 3-methyl-histidine reflects the turnover of only 65% of muscle protein and there appears to be no adequate normal standard available for comparison[244].

Body muscle mass represents the somatic mass which is one of the body protein pools, the other being the visceral protein mass. Serum or plasma proteins are measured in the hope that they will provide information about the visceral protein mass, but such measurements can be accepted only if certain assumptions are made: (i) that the measured proteins (which are liver-dependent transport proteins) reflect the composition of the visceral protein mass; (ii) that the serum concentrations reflect substrate availability (i.e. protein intake prior to assessment) rather than liver function or changes in liver mass; and (iii) that the disease process causing the nutritional impairment has no bearing on protein concentration.

The most frequently measured plasma protein in nutritional assessment is albumin, a slow turnover protein with a half life of 20 days. Transferrin is also a slow turnover protein (half life = 8-10 days), while pre-albumin and retinol binding protein are rapid turnover proteins (half life = 2 days and 10 hours respectively) and are believed by some to be better indicators of protein malnutrition than albumin and transferrin[244]. Measurement of these liver-dependent transport proteins is straightforward, rapid and inexpensive and has been used widely in epidemiological studies and nutritional studies of malnourished hospital patients.

Although low serum albumin concentrations are well documented in populations with severe and chronic protein-calorie malnutrition, albumin is a poor indicator of early or acute protein deficiency because serum levels fall slowly. Concentrations of albumin and other proteins in serum are dependent upon several factors including synthesis, catabolism, excretion and other losses, transvascular movement, administration of blood and blood products and the patient's state of hydration, and only synthesis depends on amino acid intake and availability. Albumin concentration is affected much more by changes in body fluids than by protein depletion[245]. It is because of these limitations that plasma albumin (and transferrin) measurements lack specificity and are of limited value in assessing the visceral protein compartment[244].

In spite of these inherent problems, serum or plasma proteins (especially albumin) are used widely in nutritional assessment protocols, with some justification. Plasma albumin concentration has been shown to correlate significantly with the fast turnover protein pre-albumin ($r=0.72$, $P<0.01$) in surgical patients with benign or malignant disease and requiring parenteral nutrition[245]. Serum albumin levels correlate with anthropometric methods of nutritional assessment[237] and are highly

significant predictors of malnutrition as determined by radioisotope body composition measurements[232]. However, in each of these three studies[232,237,245], the co-efficient of correlation was relatively low, suggesting that albumin does not have high specificity as a nutritional index. Serum albumin has been shown to be a predictor of post-operative morbidity and mortality[246-250]. For example, Mullen et al[236] found that patients with low serum albumin had a rate of significant complications two and a half times that of patients with normal albumin levels, although 45% of hypo-albuminaemic patients did not have complications. Klidjian et al[233] found a significant association ($P<0.001$) between low albumin concentrations and post-operative complications in 225 patients undergoing major abdominal surgery for benign (n=157) or malignant disease (n=68). As a result of this study, Klidjian recommended that a low plasma albumin be included with reduced AMC and grip strength in the definition of "appreciable protein depletion"[233].

Protein turnover studies provide a great deal more information about rates of synthesis and catabolism than single serum determinations. They provide better definition of the type and degree of malnutrition, but at present they are best regarded as research tools and cannot be used for rapid bedside diagnosis[251].

1.6.2 (d) Functional assessment

An alternative to static indices of nutritional status is assessment of physiological functions, on the basis that physical activity, work performance, cognitive ability and the response to disease are clinical expressions of functional competence which is likely to be affected adversely by malnutrition. The loss or impairment of such

functions should be more important biologically to the host than levels of a particular nutrient or protein in the circulation. A large number of functional indices of nutritional status have been described; they include tests or analyses of tissues of structural importance, host immunological responses (Section 1.6.2), nutrient transport, haemostasis and nerve function[252]. Assessment of work capacity involves testing muscle function. The ability to perform specific tasks of relatively high energy expenditure (such as bicycle ergometry) has been used to evaluate nutritional status in epidemiological studies, some of which have shown close agreement between anthropometric and functional indices and that reduction of work performance is related closely to reductions in body size and tissue mass[253,254].

Tests such as climbing, treadmill walking or bicycle ergometry are not applicable to bedside nutritional assessment of malnourished hospital patients suffering from diseases or the after-effects of anaesthesia and surgery. Klidjian et al[233,255] proposed the functional test of handgrip dynamometry as a useful non-invasive screening method of detecting malnutrition. They found that grip strength was more sensitive than anthropometric (weight for height, weight loss, AMC and forearm muscle circumference) or plasma albumin measurements in predicting post-operative complications in 102 patients undergoing major abdominal surgery. However, handgrip dynamometry lacked specificity in that it had a false positive rate of 50%, a rate similar to that of anthropometric tests[233]. The major problem with grip dynamometry and other functional tests of work capacity is that standard or normal values are required for comparison and this entails ready access to healthy age- and sex-matched control subjects. Klidjian's study used a control population of hospitalised patients (who were supposedly "healthy") and were younger than the test

population, which had a higher proportion of males[233].

The aims of functional assessment are relevant to populations of malnourished hospital patients in as much as they seek to test the capacity of the individual to respond in a physiologically normal manner to added stress. There are many inherent problems with functional tests of muscular capacity and the results of such tests are clearly influenced by motivation, physical fitness and understanding of the task required, as well as body composition. Also, exercise capacity has very wide variation in both well-nourished and malnourished subjects and so problems arise in establishing control populations and appropriate cut-off points to ensure high sensitivity and specificity. Tests such as handgrip dynamometry represent a method of functional assessment which is potentially useful to the clinician as a predictive measure of the consequences of malnutrition secondary to disease, although further confirmation of their value is awaited[256,257].

1.6.2 (e) Immunological assessment

Protein-calorie malnutrition and deficiencies of specific nutrients are associated frequently with impaired immune function (Section 1.6.1). Thus, tests of immune function have been used to quantify nutritional status. Mullen et al[236] prospectively studied the impact of nutritional status on post-operative morbidity and mortality in 64 consecutive general surgical patients: nutritional status was assessed by anthropometric (weight loss, triceps skin fold, mid-arm muscle circumference), biochemical (serum albumin and transferrin levels, protein electrophoresis, creatinine-height index) and immunological parameters (total and T lymphocyte count, serum complement level, neutrophil migration and CDHR), and patients were

monitored for post-operative complications. Serum albumin and transferrin levels and CDHR were found to be the most accurate indicators of a poor prognosis; anergic patients had a 33% complication rate, which was 2 1/2 times greater than that of patients who reacted to at least one of three skin test antigens. In another study of 162 consecutive non-emergency surgical patients, Mullen[235] used discriminant analysis and a computerised regression procedure to identify the individual power of the most important predictive variables. Again, serum albumin and transferrin levels and delayed hypersensitivity skin reactions, as well as triceps skin fold measurement, were the most useful predictive indices of complications associated with malnutrition, with serum albumin concentration being the most powerful measurement. These and other studies[258] indicate, firstly, that some parameters of immune function can be correlated with more traditional means of nutritional assessment in malnourished individuals and, secondly, that immunological assessment of malnourished patients is of some predictive value, although the specificity and sensitivity of immune function tests are not high. This second point is a feature of many of the methods of nutritional assessment.

The two immunological parameters used most widely for nutritional assessment have been CDHR ("skin testing") and the PB lymphocyte count. The value of CDHR tests in nutritional assessment of cancer patients remains unclear. Although some studies (reviewed by Hessov[259]) have been able to correlate "anergy" (or more correctly absence of an inflammatory response to intradermal injection of antigen) with significant increases in post-operative septic complications, other well conducted studies[260,261] have failed to confirm the predictive value of skin testing. Advocates of CDHR have claimed that serial skin tests are a guide to the adequacy of nutritional therapy and surgical treatment in septic patients and in

patients following major trauma[216], but the logistic difficulties of performing serial CDHR in acutely ill patients precludes use of the method in all but the most enthusiastic and well-staffed units. Although the conflicting results obtained from skin testing can be explained partly by variations in the study populations, different combinations of disease and treatment, and different criteria for grading the results, it is clear that the role of CDHR as an index of malnutrition is limited.

There are theoretical objections to the use of CDHR in the assessment of malnutrition in hospital patients. The diagnosis of impaired nutritional status by CDHR relies on the absence or reduction in size of the response to intradermal antigens. Apart from technical problems with the performance of the test and the general lack of standardisation of positive and negative responses, a negative response cannot be equated necessarily with depression of cellular immunity[59]: a CDHR has at least three distinct components - (a) an afferent component which requires sensitisation of T lymphocytes to a macrophage-processed antigen, (b) an efferent component which requires interaction of sensitised T cells with antigen and subsequent release of lymphokines, and (c) an inflammatory component which is read as a positive skin test but which is not immunologically specific as it may be induced by non-specific irritants. A defect in any component may account for an impaired skin test response. Factors suppressing the cutaneous inflammatory response (e.g. raised plasma steroid levels or acute phase reactant proteins, the plasma concentrations of which may be raised with tissue injury and in untreated cancer patients) can affect the results of CDHR. Most clinicians agree that a number of parameters should be measured when assessing the immunological status of patients, including quantitative and qualitative <u>in vitro</u> lymphocyte

studies, because dissociations between skin tests and *in vitro* lymphocyte responses are well recognised[262].

Peripheral blood total lymphocyte (PBL) counts have been used in nutritional assessment and to predict the likelihood of post-operative septic complications. A PBL count below 1.2×10^6 per ml is said to represent relative anergy as a result of moderate visceral protein depletion. The value of the PBL count as an index of nutritional impairment is not generally accepted because of its poor sensitivity. However, studies have shown that the PBL count is of prognostic significance: a retrospective survey of 105 patients found that those with lymphocyte counts of less than 1.0×10^6 per ml had significantly more post-operative sepsis than those with lymphocyte counts above this value[263]. Johnston et al[239] reported a 50% mortality among patients with subnormal lymphocyte counts, although few details of other variables were given. Others[236,260] have found this measurement to be of no prognostic significance.

A criticism of static anthropometric methods of assessment is that they reflect simply the size of a body compartment and may have little bearing on the function of that compartment or, more importantly, the ability of the host to withstand periods of inadequate nutritional intake while maintaining complete functional integrity. The same criticism can be applied to the use of the total lymphocyte count as a measure of nutritional status. The function of the immune cells would seem to be of greater importance to the host than just their number.

1.6.2 (f) Summary

It is clear from this review that no method of nutritional assessment is entirely satisfactory and that general agreement has not been

reached concerning the best protocol for assessing the nutritional status of hospitalised patients. Methods which are the most accurate and objective (e.g. TBNAA) are not suitable for use at the bedside; the most readily available and practical methods have lower specificity and sensitivity, are subject to observer error and their interpretation may be problematic (e.g. anthropometry, dynamometry); poor specificity is a feature of biochemical and immunological methods also. However, attempts have to be made to assess nutrition, and most practitioners use several techniques rather than one method alone. Until an accurate comprehensive method or index is identified, clinicians will rely on a dietary history, physical examination, anthropometry, and laboratory (biochemical) measurements. The use of functional methods is appealing but at present their value remains to be confirmed and standards for comparison are not available generally. The role of immunological methods has yet to be defined.

1.7 NUTRITIONAL THERAPY IN CANCER PATIENTS

The evidence reviewed in the previous section indicates that malnutrition is a common feature of malignancy and has wide ranging adverse effects on the immune system, and that serious complications following surgical treatment of cancer patients occur far more frequently in those who are malnourished. Current practice dictates that, in the case of many solid tumours, surgery should be performed to remove the bulk of the malignant tissue and that chemotherapy and radiotherapy be employed to eliminate residual cancer cells. Immune mechanisms may participate in the destruction of malignant cells, and so restoration of normal immune function in cancer patients could be an important factor in host resistance

against malignancy after tumour debulking. Full evaluation of the benefits of nutritional therapy in malnourished patients with cancer is still awaited.

1.7.1 EFFECTS OF NUTRITIONAL REPLETION

Although long-term studies of cancer patients who have received nutritional therapy during anti-neoplastic therapy are lacking, attempts have been made to assess the effects of nutritional repletion on body composition, immune function and short-term clinical progress in cancer patients.

1.7.1 (a) Body composition

The effect of nutritional support in cancer patients has been studied by Cohn et al[269] who reported that patients receiving total parenteral nutrition (TPN) for six weeks during chemotherapy showed increases in body weight as a result of increased body fat and water whereas the gain of protein was small; TBNAA showed that feeding had a true anabolic effect. A similar pattern was observed in patients who received enteral nutritional supplements and who gained significant (>1 kg) body weight: however, protein was lost while body fat increased in those who simply maintained their weight. In patients who lost weight during supplementation, weight loss was due largely to a reduction in total body water and a small loss of body protein: TBNAA showed that these patients had continued catabolism. Cohn et al[269] also compared standard anthropometric and biochemical indices of body composition with results obtained from TBNAA and isotope dilution techniques during periods of parenteral and enteral feeding; the anthropometric indices (AMC and TSF) correlated well with the body composition results but biochemical indices

(serum albumin and transferrin levels) and PB total lymphocyte count did not.

In summary, some evidence shows that nutritional repletion leads to increased body weight, but the functional value of this is dubious because the increases result largely from retained body water with little improvement in body protein. However, lean body tissue, subcutaneous fat and whole body protein synthesis can be increased in some patients.

1.7.1 (b) Immunological parameters

Nutritional repletion with enteral or parenteral feeding has been shown to restore some of the immmune deficits associated with malnutrition. Haffejee et al[270] used enteral feeds to correct protein-calorie malnutrition in 15 patients with inoperable carcinoma of the oesophagus; increases in total and T lymphocyte numbers and <u>in vitro</u> lymphocyte reactivity occurred after achieving a positive nitrogen balance. Interestingly, improvement in immunological parameters occurred without any attempt at therapeutic reduction in tumour bulk, a finding which adds weight to the conclusions of Dominioni et al[220] that malnutrition, rather than the presence of malignancy, is the major factor in the impaired immunological parameters found in many malnourished cancer patients. Enteral feeding of malnourished animals has been shown to restore depressed cellular and humoral immunity[271] and to reverse their increased susceptibility to bacterial and viral infections[272].

Dionigi et al[273] demonstrated in dogs with subacute malnutrition that TPN with fat-free solutions resulted in a return to normal of neutrophil chemotaxis and serum concentrations of IgG, IgM and C3, and that the primary immune response of the dogs to thymus-dependent antigens was

stronger and more rapid following TPN. In contrast to earlier findings[274], treatment of the dogs with TPN did not restore *in vitro* proliferative responses of lymphocytes to mitogenic stimulation. This result, which is at variance with those of others[258,275,276], was thought to be due to either the relatively short period of treatment (21 days) or the persistent stress of having permanent indwelling central venous catheters and being constrained constantly in metabolic cages.

Law et al[275] showed in 7 malnourished patients with benign diseases that intravenous feeding with fat-free solutions for 18 days resulted in significant improvement of *in vitro* lymphocyte proliferative responses to T cell mitogens, although humoral immunity was not affected. Copeland et al[276] reported that prolonged parenteral nutrition with fat-free solutions in malnourished cancer patients resulted in improvement of CDHR - 19 of 33 patients with initial negative skin responses converted to positivity during parenteral feeding. Significantly, of patients undergoing surgery, all with negative skin responses either died or had prolonged post-operative complications, whereas two-thirds with positive skin responses had uncomplicated post-operative courses. Of patients receiving chemotherapy, tumour regression was seen only in patients whose skin tests converted from negative to positive during intravenous feeding or who had positive skin tests before intravenous feeding commenced.

It is evident, therefore, that nutritional repletion can reverse some immunological abnormalities observed in malnourished cancer patients. These findings give indirect support to the use of immunological parameters in nutritional assessment of cancer patients, although the limitations of such assays must be borne in mind (Section 1.6.2).

1.7.1 (c) Clinical results

Weight loss in patients with cancer has been identified as a factor associated with decreased survival[277] and several studies have been undertaken to evaluate nutritional therapy as an adjunct to antineoplastic therapy. Generally, such studies have attempted to answer two questions: does nutritional therapy influence the outcome of antineoplastic chemotherapy or radiotherapy, and does nutritional therapy influence the morbidity and mortality of surgery in cancer patients?

A summary of seven randomised clinical trials of TPN in addition to chemotherapy is given in Table 1.4. No significant improvement in survival or response to treatment was associated with the use of TPN in addition to chemotherapy in any of these studies of patients with advanced cancer, but it should be noted that the number of patients in each study is small, with the exception of the study by Clamon et al[280]. When considered together, the results are consistent with the view that provision of calories and amino acids does not alter the clinical course of patients receiving anti-cancer chemotherapy. Parameters such as patient wellbeing were not considered by many of these studies, which may have included too few patients or provided nutritional support for too short a time for major benefits to be obvious. Similarly, TPN appears to have no place in the routine management of cancer patients undergoing radiotherapy[285]. However, that is not to say that malnourished patients will not require some form of nutritional support during their radiotherapy if they are unable to eat or drink nutritious substances.

The second major area of interest has been the value of short-term nutritional support in preventing complications following surgical treatment of malignancies. A summary of seven randomised trials of surgery with or without TPN is given in Table 1.5. Again, these studies[286-292]

Table 1.4 Summary of seven randomised trials of TPN in addition to chemotherapy in patients with advanced cancer

Tumour	Number of patients	Duration of TPN (days)	Result	Ref.
Colon	45	8-36	Increased weight and significantly decreased survival with TPN	278
Lymphoma	36	14	NSD in chemotherapy tolerance	279
Lung	119	28	NSD in survival or response rate	280
Lung	49	42	Higher (NS) response rate, maintenance of body weight and significant improvement in CDHR with TPN	281
Lung	19	34	NSD in response rate or haematological complications	282
Testis	30	48	NSD in response rate or chemotherapy side effects; significantly less weight loss and significant increase in infections with TPN	283
Sarcoma	32	7	NSD in response rate or long-term survival	284

TPN = Total parenteral nutrition

NSD = No significant difference

NS = Not significant

Table 1.5 Summary of seven randomised trials of TPN in addition to surgery in patients with surgically resectable cancer

Tumour	Number of patients	Timing and duration of TPN (days)	Result	Ref.
All GI	127	Pre-op, 10	Significant reduction in mortality and major complications and N.S. reduction in wound infections with TPN	286
GI	26	Pre-op, 12	NSD in survival or complications	287
GI	56	Pre-op, 13	NSD in survival or complications. Reduced post-op weight loss with TPN	288
Upper GI	74	Pre and post-op 7-15	NS reduction in mortality and morbidity and significant reduction in wound infection with TPN	289
Oesophageal	15	Pre and post-op 14	NS reduction in complications and better wound healing with TPN	290
Oesophageal	20	Pre-op, 28	NS reduction in post-op morbidity, greater weight gain and earlier positive N balance with TPN	291
Gastric	70	Pre-op, 7-10	Significant reduction in wound infections and NS reduction in mortality and anastomotic leak rate with TPN	292

N = Nitrogen

NS = Not significant

NSD = No significant difference

TPN = Total parenteral nutrition

involved small numbers of patients and the periods of nutritional support were short. Most studies showed some benefit of TPN in patients with operable cancer of the gastrointestinal tract although differences between test and control groups did not always reach statistical significance.

1.7.1 (d) Potentially adverse effects of intravenous nutrition

Intravenous nutrition requires central venous catheterisation which exposes patients to many potential complications related to infection, venous thrombosis and mechanical problems with the catheters, connector pieces, intravenous lines and delivery systems[293]. In addition to mechanical and metabolic problems, reservations have been raised about the use of nutritional support for cancer patients because of the possibility that the tumour might sequester nutrients for its own growth and thereby benefit disproportionately in comparison with host tissues. Carmichael et al[268] showed in humans that protein was translocated from protein stores (such as muscle) to tumours where protein synthesis was rapid and greater than that of host tissues. Stein et al[294] found that tumour-bearing rats retrieved more nitrogen than control rats on the same dietary intake and that the tumours demonstrated a rapid rate of protein synthesis on all but the most restrictive diets; even on deficient diets the rate of protein synthesis in tumours was greater than that of any other tissue. White[295] noted that protein-energy malnutrition inhibits tumour growth in rodents and increases the tumour latency period after implantation.

Other workers have reported stimulation of tumour growth by nutritional repletion of protein-depleted animals. Popp et al[296] demonstrated in Fisher rats with sarcoma transplants that tumour weight increased more than the host lean carcase weight as the rate of calorie infusion increased. No evidence has appeared as yet to indicate that

nutritional support stimulates tumour growth in humans[297].

1.7.2 INTRAVENOUS NUTRIENT SOLUTIONS AND IMMUNE FUNCTION

A further source of insult to the cancer patient receiving TPN is the intravenous fluids themselves, apart from any effect which they may have on nutritional status. When evaluating the effects of intravenous nutrition, account must be taken of the effects of the intravenous nutrient solutions themselves, in addition to effects brought about by improved nutritional status. This is of particular importance when intravenous nutritional therapy is given only for a short time, when improvements in nutritional status may not occur. For example, if an intravenous solution was found to have a detrimental effect on immune function, it would not be advisable to use it in patients with proven reduced immunity because the immune system would be impaired further before any beneficial effect of improved nutrition was evident.

The studies of parenteral feeding of malnourished cancer patients (Section 1.7.1) showed improvement of immunological parameters and some improvements in post-operative morbidity and mortality[273,274,276,286-292]. Interestingly, all of these studies used combinations of amino acid and carbohydrate solutions as protein and energy sources respectively, and none used fat emulsions.

The apparent absence of fat emulsions in many studies of hyperalimentation is probably due to their relatively late appearance in the USA, where most studies have been performed, and to the widely held belief that fat is inferior to glucose as an energy source. With the recognition that fat emulsions have some definite advantages over carbohydrate as sources of energy[298,299], it is probable that they will

be used more widely and included in studies of parenteral nutrition.

Fat emulsions suitable for clinical use were produced first in Japan in the 1920's and have become accepted components of TPN regimes. They contain essential fatty acids and provide relatively large amounts of calories in small isotonic volumes. Most fat emulsions contain only a small proportion of free fatty acids but have a high content of esterified polyunsaturated fatty acids (PUFA). For example, oleic, linoleic and linolenic acids account for 86% of the esterified unsaturated fatty acids in "Intralipid" (Kabivitrum Ltd, Stockholm), the most widely used commercially available fat emulsion. Fatty acids exist normally as either triglycerides or phospholipids, and similarly, free fatty acids comprise less than 1% of commercially available fat emulsions.

There are several reports concerning the immunosuppressive effects of PUFA. Mertin et al[300], in a comparative study of five fatty acids (palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and arachidonic acid (C20:4)) found that in vitro transformation of normal human lymphocytes was inhibited by all of these fatty acids at concentrations equivalent to and greater than physiological concentrations. The inhibitory effects were dose-related, with oleic acid being the most potent inhibitor; among the unsaturated fatty acids, there was a stepwise increase in inhibitory activity (arachidonic < linoleic < oleic). PUFA showed a significant difference between inhibition of transformation of stimulated (with PPD and PHA) and non-stimulated lymphocytes[300], suggesting that PUFA have a specific inhibitory action restricted to activated lymphocytes, whereas the inhibitory effects of saturated fatty acids were seen with stimulated and non-stimulated lymphocytes, suggesting non-specific inhibition. Mertin et al[301] found that linoleic and arachidonic acids significantly inhibited in vitro reactivity of human

lymphocytes to PPD, and that inhibition occurred only when PUFA were added to lymphocytes before but not after antigen was added, suggesting PUFA interfered with recognition of antigen by lymphocytes. Similarly, Offner et al[302] found that oleic, linoleic and arachidonic acids and prostaglandin E1 and E2 at physiological concentrations significantly inhibited lymphocyte activity, with the same stepwise increase in inhibitory activity observed by Mertin et al[300,301].

The inhibitory effects of PUFA on cellular immune responses have been demonstrated in vivo also. Ring et al[303] demonstrated that survival of skin allografts in rats was prolonged significantly by oral or intraperitoneal administration of linoleic acid. Kort et al[304] demonstrated significant prolongation of kidney allograft survival by feeding rats with a diet rich in sunflower seed oil which contained about four times the amount of linoleic acid given to control animals. Similar results have been obtained in mice with skin allografts[305]. The immunosuppressive properties of linoleic acid have been explored in human cadaveric renal transplantation by the use of sunflower seed oil in addition to conventional immunosuppressive therapy in recipients of renal allografts[306].

The demonstration that saturated and unsaturated fatty acids have immunosuppressive properties is of concern to clinicians who might prescribe fat emulsions for nutritional therapy in clinical practice. Patients who are candidates for parenteral nutritional support frequently have impaired cellular immunity and so the possibility arises that the use of fat emulsions, with their high content of fatty acids, could result in further suppression of cellular immunity, thereby exposing patients to increased risks of septic complications. Although evidence shows that

restoration of nutritional status by prolonged intravenous feeding with fat-free fluids can result in improvement of immune function, the short-term effects of the intravenous nutrients themselves, especially those containing fats, are unknown. Thus, the effects of intravenous nutrient solutions on lymphocyte activity were studied and the results reported in Chapter 4.

1.8 HYPOTHESES

It is apparent from the reviewed literature that the immune system is a highly complex integrated system which has far reaching influences in the intact human and which itself is influenced by regulatory systems and mechanisms. The cell of central importance is the lymphocyte. The reactivity of individual components of the immune system can be impaired by many factors and disease states, including malignant disease and malnutrition.

The paradox of progressive tumour growth in the presence of specific anti-tumour humoral and cell-mediated immune responses has led to the concept of "immunological escape" of tumours from the control and influence of their host. One of the many abnormal findings in cancer patients is the presence of immunosuppressive factors in plasma. These factors are poorly defined, and their importance and contribution to the impairment of immune competence and to the growth of tumours is unknown. It has been pointed out that tumours release many molecules from their constituent cell surfaces, including protease enzymes (P), and that alpha-2-macroglobulin (A2M), a plasma protease inhibitor, has the ability to eliminate proteases, with the result that a relatively immunosuppressive A2M-P complex is formed.

The first hypothesis of this study is that the presence of

malignant disease is associated with factors which are non-specifically immunosuppressive and which, having been produced or induced by a tumour, cause the plasma of patients with cancer to have increased suppressive activity compared with that of normal subjects; in view of the wide ranging activities of A2M, it is proposed that A2M has an important role in the immunoregulation of cancer patients and that this naturally occurring molecule has a different or additional biological function in such patients. If this hypothesis is correct, then modification of a naturally occurring "homeostatic" molecule by the presence of a tumour or tumour-derived factors could represent another mechanism of immunological escape.

It is evident from the reviewed literature that malnutrition impairs host immunity. Interest in the adverse effects of malnutrition has centred largely on cellular defects and very little is known about changes in humoral components apart from the immunoglobulins. In view of the wide ranging and relatively non-specific nature of the immune deficit found in malnourished subjects, the second hypothesis proposes that non-specific immunosuppressive factors are present in the plasma of such subjects and may be responsible in part for their immune deficit.

The problems of nutritional assessment have been reviewed and it is clear that no single method exists to satisfactorily classify or quantify the malnourished state. The value of functional as opposed to static nutritional parameters has been considered. Malnourished patients have a high incidence of infections and so researchers have attempted to correlate tests of immune function with the more traditional methods of nutritional assessment. This experimental work will determine whether a correlation exists between a new parameter, the lymphocyte suppressive activity in a subject's plasma, with standard well-accepted anthropometric methods of nutritional assessment.

Routine intravenous nutritional support of cancer patients undergoing surgery or chemotherapy has been shown to be of dubious value in terms of survival, increasing tolerance to chemotherapy or reducing the morbidity of anti-neoplastic therapy. Several studies have shown that intravenous feeding with fat-free fluids improves tests of immune function. Modern TPN regimes usually include emulsions of fatty acids, which have immunosuppressive properties *in vitro*. Thus, the third hypothesis is that such fat emulsions will adversely affect host immune function, and this has been investigated by experimental studies.

The hypotheses have been examined by experiments with the following aims:

1. To measure *in vitro* lymphocyte reactivity and the non-specific lymphocyte suppressive activity of plasma in patients with malignant or benign disease and in healthy subjects.
2. To determine if *in vitro* lymphocyte reactivity and the lymphocyte suppressive activity are abnormal in malnourished individuals, and if so, to identify any correlation between these parameters and conventional methods of nutritional assessment.
3. To identify the factor or factors in plasma associated with measurable lymphocyte suppressive activity.
4. To define the role of A2M with respect to lymphocyte reactivity and the suppressive activity of plasma in the above mentioned subjects.
5. To assess the relationship between the suppressive activity of plasma and the growth of tumours, using an animal model.
6. To measure the effect of some intravenous nutrient solutions in common use on *in vitro* reactivity of lymphocytes and to measure lymphocyte responsiveness in patients receiving these fluids.

CHAPTER 2

MATERIALS AND METHODS

2.1 EXPERIMENTAL SUBJECTS

There were three groups of experimental subjects in this study: (i) healthy volunteers, (ii) patients with benign conditions, and (iii) patients with malignant disease. Healthy volunteers were members of staff of either the Department of Surgery, University of Newcastle upon Tyne, or the Royal Victoria Infirmary, Newcastle upon Tyne, England. The patients were in-patients receiving treatment on the Professorial Surgical Unit, Royal Victoria Infirmary. All subjects agreed to withdrawal of 20 ml of venous blood specifically for this research; some patients kindly donated blood on more than one occasion.

A diagnosis was established for each patient by traditional methods. No clinical investigation was performed purely for the sake of the project. Details of history, physical examination, investigations and nutritional assessment were recorded by the author on a specially designed proforma. Patients were excluded from the study if a firm diagnosis could not be made. All patients underwent surgery, some being operated upon by the author. Confirmation of the diagnosis was made at operation and histopathological examination was made of any excised tissue.

The details of subjects used in different parts of the study are given within relevant sections.

2.1.1 PATIENTS WITH MALIGNANT DISEASE

Sixty-nine patients with malignant disease were studied. The primary sites of malignant disease are shown in Table 2.1. Tumours were staged according to the TNM classification[307] and assigned to one of four clinical stages which are used widely in clinical practice (Appendix 1). Attempts were made to determine accurately the extent of disease in each patient by: (i) pre-operative investigations such as ultrasonography and

Table 2.1 Sites of primary tumours in patients with malignant disease

PRIMARY SITE	MALE	FEMALE	TOTAL
Colon or rectum	20	16	36
Breast	0	14	14
Stomach	8	4	12
Pancreas	2	5	7
Total	30	39	69

isotope scintigraphy, (ii) operative assessment by the surgeon by visual inspection and palpation of regional lymph nodes and relevant viscera, and (iii) histopathological examination of resected tissue by pathologists in the Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne, England.

2.1.2 PATIENTS WITH BENIGN DISEASE

Fifty-two patients with symptomatic benign diseases were studied (Table 2.2). Diseases were of such severity that surgery was required. No attempt was made to match patients in the benign and malignant groups in terms of matching one disease process with another, although attempts were made to match symptoms and the tissues or viscera which were the sites of disease. This has relevance to clinical situations: the clinician may be faced with a patient who has a symptom complex compatible with either a benign or malignant disease, or the site of disease may have been localised by examination or investigation but the nature of the disease remains unknown. The age and sex distributions of the two groups were similar.

2.2 NUTRITIONAL ASSESSMENT

The methods of nutritional assessment used in the present study were body weight-for-height, weight loss expressed as a percentage of usual or pre-illness weight, standard anthropometric measurements of skin fold thickness and arm muscle circumference, and serum albumin. These methods were chosen because of the availability of reference standards, and the ease with which they could be used repeatedly in sick immobilised patients.

Nutritional assessment of patients was performed on admission to hospital before any form of treatment was undertaken. Anthropometric

Table 2.2 Diagnoses of patients with benign disorders

DIAGNOSIS	MALE	FEMALE	TOTAL
Cholelithiasis	2	13	15
Diverticular disease	6	6	12
Inflammatory bowel disease	4	2	6
Peptic ulceration	3	1	4
Chronic pancreatitis	4	0	4
Benign breast disease	0	4	4
Oesophageal stricture	2	1	3
Haemorrhoids	2	0	2
Gastro-intestinal fistula	2	0	2
Total	25	27	52

assessments were performed by the author.

2.2.1 WEIGHT-FOR-HEIGHT

Patients were weighed on a standard hospital beam balance scale while wearing only pyjamas. Most patients were weighed by the nursing staff after instruction by the author who undertook the weighing of some patients. Patients' weights ("present weight" - PW) were recorded in kilograms. The height of each patient was measured using a metric vertical wall ruler with the patient standing upright without shoes. Patients who were immobilised in bed on admission were measured before discharge from hospital.

The ideal body weight (IBW) for height and sex was obtained from the Metropolitan Life Insurance Company tables adapted by Jelliffe[230]. Each patient's PW was compared with his or her IBW by the ratio PW/IBW which was expressed as a percentage.

2.2.2 WEIGHT LOSS

Assessment of weight loss was made by comparing each patient's PW with his or her pre-illness or usual weight (UW), using the formula $\frac{UW - PW}{UW}$ and expressing the result as a percentage. The value of UW was obtained from medical records or the patient's own documentation, or by patient recall. The potential inaccuracies of patient recall of weight loss have been acknowledged (Section 1.6.2) but had to be accepted for the purpose of the study.

2.2.3 ARM MUSCLE CIRCUMFERENCE

Arm muscle circumference (AMC) was used to assess lean body mass. The arm was assumed to be spherical (Figure 1.1) and AMC was calculated by

correcting the whole arm circumference measured at the mid point of the arm (mid arm circumference ; MAC) for the subcutaneous fat layer as measured by the triceps skin fold (TSF) thickness, using the formula :

$$AMC = MAC - (\pi \times TSF)$$

Measurements were performed by firstly identifying the mid-point of the non-dominant arm as the point mid-way between the tip of the acromion process of the scapula and the tip of the olecranon process of the ulna with the elbow joint flexed at 90 degrees. The non-dominant arm was used for all measurements as it was considered that its size would be less likely to be affected by variations in physical activity and exercise. The circumference of the arm at the mid-point was measured three times in centimetres and the mean value recorded. AMC values for each patient were compared with standards for healthy males or females[230] and expressed as percentages of these standards (AMC %).

2.2.4 TRICEPS SKIN FOLD THICKNESS

Fat stores were assessed by measurement of the triceps skin fold (TSF) thickness of the non-dominant arm. With the patient's elbow joint extended, the skin fold immediately above the mid point of the arm was picked up gently between thumb and index finger, lifting the skin and subcutaneous fat away from the underlying triceps muscle. Spring calipers (Holtain Ltd., Dyffed, UK) were applied to the skin fold at the mid point of the arm (Figure 1.1). The tension of the spring was such that it did not compress the skin or fat unduly and the procedure was not uncomfortable. The TSF was measured three times and the mean value recorded.

2.2.5 SERUM ALBUMIN

The concentration of albumin in serum was used as a measure of visceral protein[108]. Estimations were performed in either the Department of Clinical Chemistry at the Royal Victoria Infirmary, Newcastle upon Tyne, using a colorimetric auto-analyzer, or by the author using single rocket immunoelectrophoresis (Section 2.6).

2.3 THE TANNED ERYTHROCYTE ELECTROPHORETIC MOBILITY (TEEM) TEST

The TEEM test was used throughout this study to measure *in vitro* lymphocyte reactivity. The TEEM test measures the effect of soluble lymphokine on the electrophoretic mobility of indicator particles. Interaction of lymphocytes with an antigen or mitogen releases soluble lymphokine which alters the normal surface electric charge of tannic acid-treated sheep erythrocytes, which simply act as indicator cells; the change in surface charge alters the mobility of the tanned sheep erythrocytes within the electric field of the cytopherometer, which is the instrument in which movement of the sheep erythrocytes can be observed and measured.

2.3.1 BACKGROUND

Detection of *in vivo* cell-mediated immunity (CMI) has relied traditionally on CDHR. A positive result requires that the subject is pre-sensitised to the antigen and that the afferent and efferent limbs of the immune response and components of the inflammatory response are intact, a situation which may not exist in patients with cancer or nutritional deficiency[59]. There are many inherent problems with CDHR tests, not the least of which is their actual performance. The advantages of *in vitro* tests of CMI include the potential to examine and quantify just one

component of the series of events which make up CDHR, ability to control test conditions (and thereby eliminate many of the problems and sources of error of in vivo tests), and the relative ease with which in vitro tests can be performed.

The first in vitro assay system which correlated with delayed hypersensitivity reactions was described in 1922 by Holst[308] who observed that the migration of human white blood cells was sometimes inhibited after exposure to tuberculin. Rich et al[309] in 1932 showed in vitro that tuberculin significantly inhibited the migration of spleen cells and PB white cells of guinea pigs immunised with non-virulent human tubercle bacilli. Since then, several in vitro assay systems have been developed to examine cell-mediated immunity.

The Macrophage Migration Inhibition (MMI) test was described by David et al[310] in 1964 and became an accepted in vitro method of assessing delayed hypersensitivity reactions. David observed that the migration from capillary tubes of cells obtained from peritoneal exudates of guinea pigs sensitised to PPD was inhibited consistently and markedly by small amounts of PPD. Later, it became clear that lymphocytes in the peritoneal exudate were the immunologically active cells and that the sensitised lymphocytes after in vitro contact with the sensitising antigen (PPD) elaborated a soluble material capable of inhibiting migration of the macrophages[311], which were simply indicator cells. Diengdoh and Turk[312] found that the electrophoretic mobility of peritoneal exudate macrophages from tuberculin-sensitised animals was altered by the supernatant of the lymphocyte-antigen interaction; they concluded that a soluble factor (or factors) liberated from stimulated lymphocytes altered the surface electrostatic charge of macrophages, thereby altering their

electrophoretic mobility.

2.3.1 (a) Cell electrophoresis

Electrophoresis means the movement of particles with a net electric charge in a solution under the influence of an electric field. Reuss[313] in the early nineteenth century observed the movement of kaolin particles towards the cathode when an electric current was passed across a tube containing water, sand and kaolin. The theoretical basis of electrophoresis was defined by Helmholtz[314] in 1879 and Smoluchowski[315] in 1903: they reasoned that the migration velocity of a particle within an electric field depended on the electric charge of the particle, the size of the electric field and the properties of the medium containing the particle. The Helmholtz-Smoluchowski equation describes the electrophoretic mobility of a particle :

$$u = \frac{E \times D}{4 \prod n}$$

u = electrophoretic mobility (microns/second/volt)

E = potential difference between the surface of shear and the suspending medium

D = dielectric constant of the suspending medium

$\prod$ = constant (3.14159)

n = viscosity of the suspending medium

Cell electrophoresis was used almost solely as a research technique to investigate electric potentials at cell surfaces until the 1960's. Electrophoretic techniques have been applied to immunological research since 1967 when Sundaram et al[316] showed that the electrophoretic mobility of sensitised lymphocytes was reduced significantly following re-

exposure to the sensitising antigen. Thereafter, tests utilising the electrophoretic mobility of cells were applied widely to immunological studies, such as the investigation of human blood groups[317], specificity of antibody production by lymph node cells[316] and monitoring of absorption of viruses and antibodies to cell surfaces[318,319].

2.3.1 (b) The Macrophage Electrophoretic Mobility test

Field et al[320] applied an electrophoretic method to the MMI test, and demonstrated that incubation of sensitised PBL with the sensitising antigen in the presence of irradiated guinea pig peritoneal exudate macrophages resulted in reduction of the electrophoretic mobility of the macrophages, which acted as indicator "particles" in the electrophoretic chamber. This single step test, the Macrophage Electrophoretic Mobility (MEM) test, provided a rapid in vitro quantitative assessment of lymphocyte reactivity[320].

The MEM test has been compared with established in vitro tests of cell-mediated immunity by Mertin et al[321] who demonstrated a very close correlation between results obtained with the MEM test and those from classical lymphocyte transformation. Hughes et al[322] compared the MMI test with the MEM test in studies of guinea pigs sensitised to encephalitogenic factor, a brain extract; they found a highly significant correlation between the two tests and noted that the MEM test was readily repeatable and of greater value than MMI in estimating the degree of sensitivity to the stimulating antigen. Others[323] have made similar observations.

Criticisms of the MEM test have been aimed mainly at the method. The possibility of a mixed lymphocyte reaction occurring between human test lymphocytes and lymphocytes present in the guinea pig peritoneal exudate

was an obvious source of error, which was partly overcome by irradiating the peritoneal exudate with 100 Rads[320]. To remove the possibility of a mixed lymphocyte reaction interfering with the lymphocyte-antigen reaction, Pritchard et al[324] introduced a two stage MEM test or "modified MEM" (MOD-MEM) test, so that there was no contact between test lymphocytes and any cells in the guinea pig peritoneal exudate. Excellent correlations between the MEM and MOD-MEM tests have been demonstrated[324,325].

Most of the difficulties relating to operation of the MEM and MOD-MEM tests were due to the production and intrinsic characteristics of guinea pig macrophages which could not be stored, and had low and somewhat variable electrophoretic mobility. These problems were overcome by Porzsolt et al[326] who showed in 1975 that sheep red blood cells (RBC), stabilised with tannic acid, could be used as indicator particles in place of guinea pig macrophages for detecting lymphokine slowing factor. This improved test, using tanned sheep erythrocytes, is the Tanned Erythrocyte Electrophoretic Mobility (TEEM) test[326,327]. Substitution of tanned sheep RBC for peritoneal macrophages as indicator cells has several advantages. Firstly, RBC are extremely homogeneous with respect to their electrophoretic mobility and so any alteration of their mobility within the test system is obvious. Secondly, RBC have good stability in the cytopherometer and are not subject to the same rapid sedimentation as macrophages. Thirdly, sheep RBC are readily available and inexpensive, and the method of preparation is simple (Section 2.3.3(c)).

Shenton et al[327] showed clearly that the kinetics of the TEEM test closely resemble those of the MEM and MOD-MEM tests with respect to (a) antigen response curves for the interaction of human lymphocytes with PPD antigen; (b) lymphocyte number response curves with PPD as the test

antigen; (c) the effect of diluting slowing factors on electrophoretic mobilities of indicator cells; (d) the temperature-time response curve for the first stage of the MOD-MEM and TEEM tests; and (e) the temperature-time response curve for the second stage of the MOD-MEM and TEEM tests. The active components in the supernatant of lymphocyte-antigen interactions (i.e. macrophage- and tanned sheep erythrocyte-slowing factors) have been characterised, and have molecular weights between 13,000 and 15,000 daltons and have similar gel filtration patterns. The results obtained with the MEM and TEEM tests for mixed lymphocyte reactions and lymphocyte reactivity to PPD correlate well[327]. More recently, the TEEM test has shown excellent correlation with the classical lymphocyte transformation assay in measuring lymphocyte sensitivity to cortisol[328]. The principle of the TEEM test is shown in Figure 2.1.

With the description of the MEM test in 1970 came immense interest in the possible application of cell electrophoresis to clinical medicine. Perhaps greatest enthusiasm was centred around the use of electrophoretic mobility tests in the diagnosis of malignant disease. In Field's original description of the MEM test[329] he reported that lymphocytes sensitised to a basic protein extracted from brain tissue, encephalitogenic factor, occurred not only in patients with carcinomatous neuropathy but also in subjects with malignant tumours without neurological damage. Further studies by Field et al[330,331] confirmed the early findings and extended them to demonstrate that the test could identify many individuals with malignant tumours, irrespective of site or extent, as well as those who had undergone surgical excision of a malignant growth and were apparently disease-free. Although the value of the MEM test in detecting the presence of malignancy by measuring lymphocyte sensitisation was confirmed by centres throughout the world[323-325,332-340], it did not

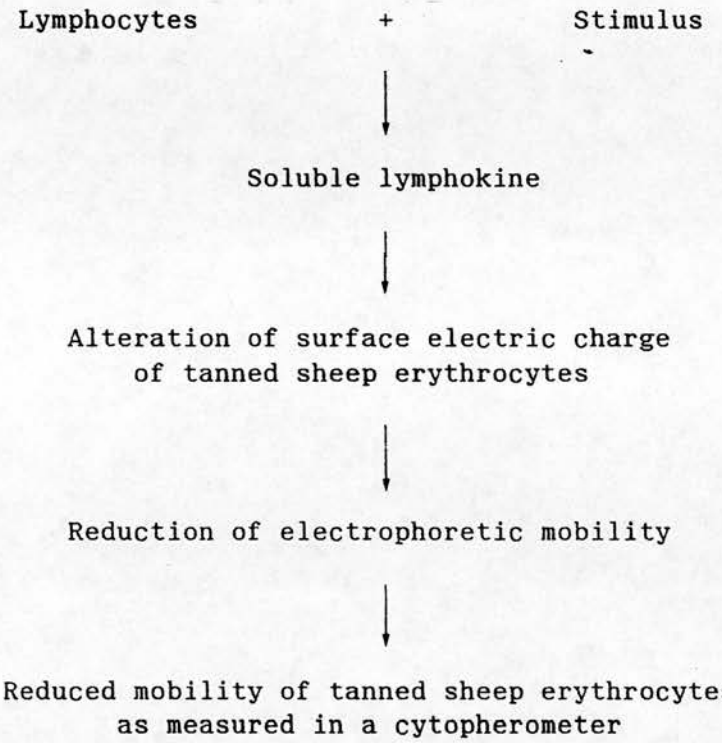


Figure 2.1 The principle of the tanned erythrocyte electrophoretic mobility (TEEM) test

achieve general acceptance.

Cell electrophoresis has also been used in the investigation of subjects with suspected neurological diseases[320] and in the measurement of mixed lymphocyte reactions as a prognostic factor in human kidney transplantation[341]. The TEEM test has been used extensively in clinical organ transplantation[342] and cancer research[343,344].

In spite of an extensive literature relating to the use of cell electrophoresis (and the TEEM test in particular) in clinical medicine, the technique has not been accepted widely. The technique was seen in a poor light after early and often extravagant claims for its value in cancer diagnosis were not substantiated. However, such claims were made by a small group of enthusiasts and reflected on the application of the technique, not the technique itself. The two major limitations of the TEEM test are the requirement of expensive equipment in which to perform the test and the laborious nature of the performance of the test. Fortunately, the equipment necessary for the TEEM test was available in the laboratory in which the present work was performed, and the tests were performed by a recognised expert in the field, Dr. Brian Shenton.

2.3.2 THE CYTOPHEROMETER

The cytopherometer (Carl Zeiss, GDR) is the machine in which the TEEM test is performed (Figure 2.2). It consists of an electrophoretic chamber, in which cells migrate under the influence of an electric field, and an optical system for observation of cell movements (Figure 2.3). The chamber has a volume of 3.0 ml which determines the volume of each sample to be tested. Samples are introduced into the right hand side of the chamber and eluted from the left. The chamber is surrounded by a water bath and maintained at a constant temperature of 23^{o}C by a thermostat. The

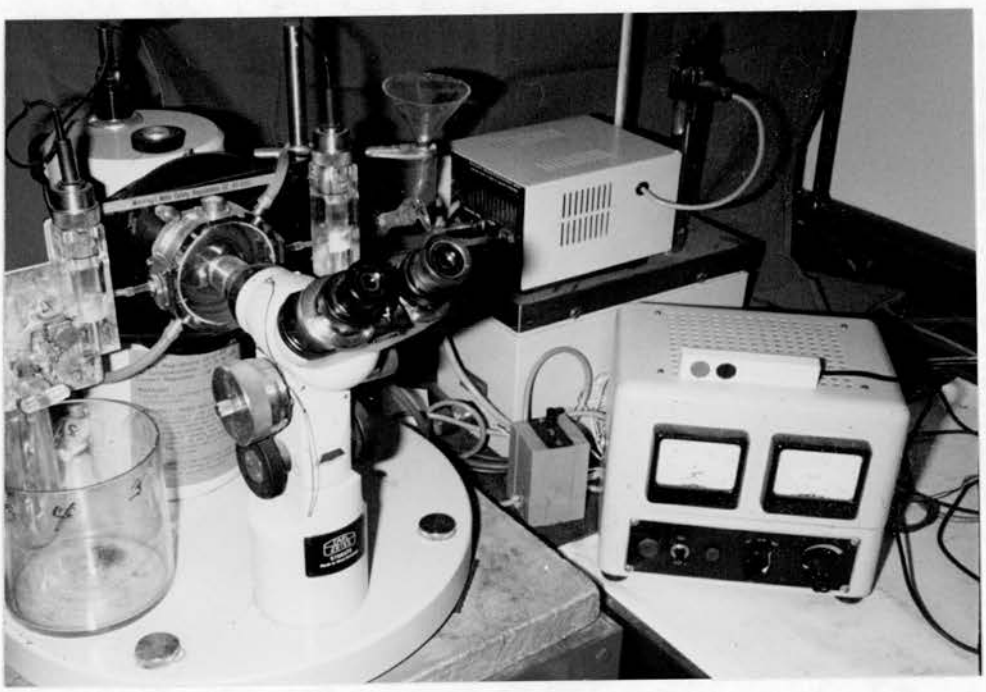


Figure 2.2 The cytopherometer

DIAGRAM OF CYTOPHEROMETER

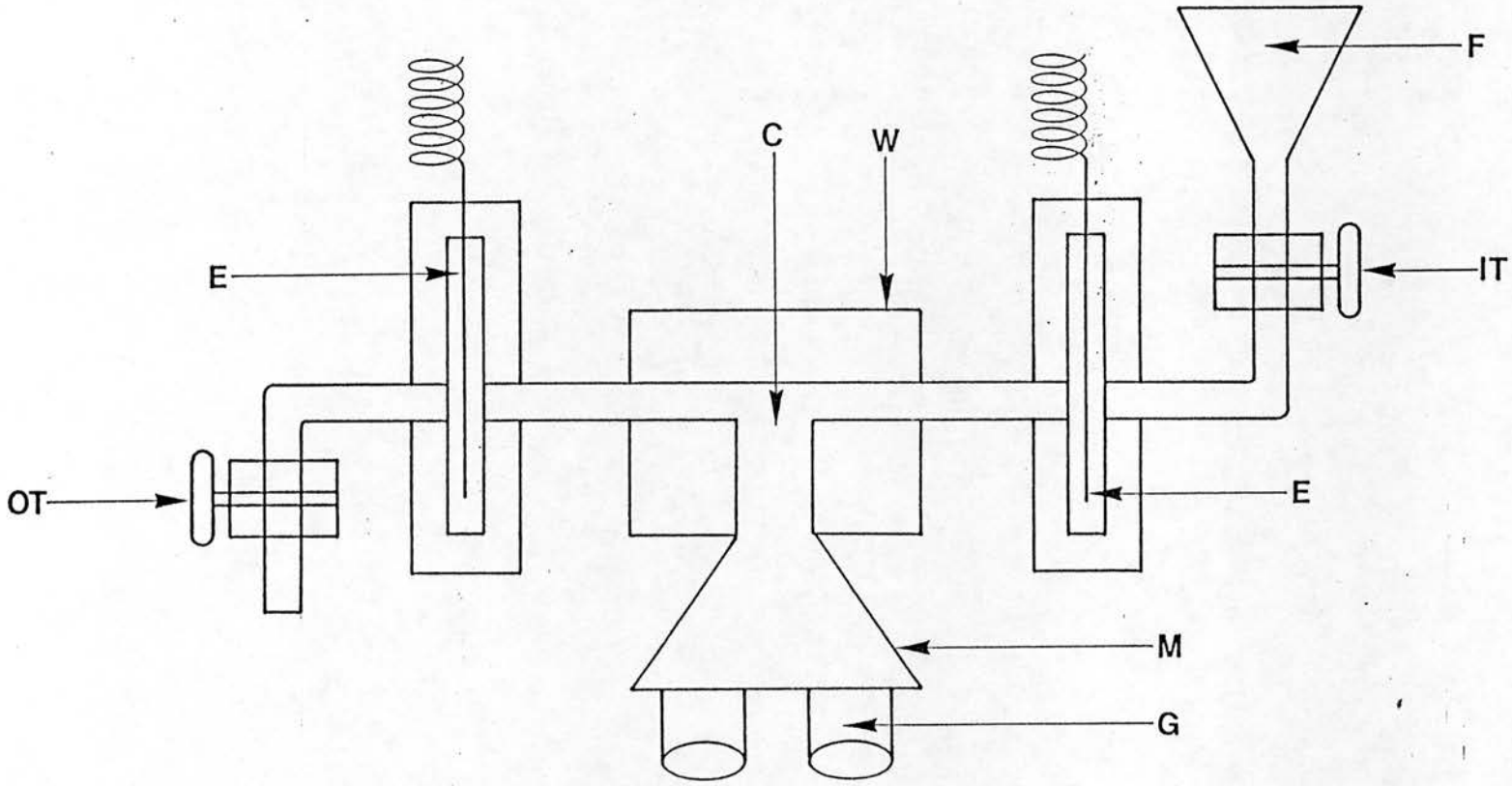


Figure 2.3 Diagram of cytopherometer

C = Chamber, E = Electrodes, M = Phase contrast microscope,
G = Graticule, W = Water jacket, F = Filter funnel,
IT = Tap controlling the inflow into the cytopherometer
OT = Tap controlling outflow from cytopherometer

electrodes, which are platinum wire covered electrolytically with a layer of silver chloride, are isolated from the chamber by glass sinters.

Cells in the chamber are visualised by a phase contrast microscope. In the right-sided eyepiece of the cytopherometer is a graticule composed of one hundred 16 micron squares. The visual field seen on looking into the cytopherometer is illustrated in Figure 2.4.

Samples to be tested were introduced into the chamber through the filter funnel and the tanned sheep red blood cells (TSRBC) were allowed to recover from the turbulence of introduction for a short time before measuring their electrophoretic mobility.

The viewing microscope was focused on the stationary layer of the electrophoresis chamber and only cells in sharp focus were measured. There was a slow constant upward drift of cells when observed through the microscope eyepiece, due to an inverted image of gravitational sedimentation. A constant electric current of 7.5 milli amps was applied between the electrodes at 180 volts and cells in the chamber moved towards the cathode. Cells were timed in one direction between the two vertical lines of one of the 16 micron squares in the eyepiece grid, and then each cell was made to move in the opposite direction by reversing the current. The time taken to move in both directions was measured by a digital electronic timer. Any cell showing more than a 10% difference in velocity for the two directions was excluded. Ten cells were counted for each sample.

The following is an example of the times obtained for 10 TSRBC to move in both directions, that is over the 32 micron distance: 6.01, 5.96, 5.91, 5.98, 6.03, 6.13, 5.89, 6.00, 5.93, 5.97. Mean time ± 1SD = 5.981 ± 0.065 seconds. After measuring and recording the times for 10 cells, the sample was eluted from the left side of the chamber which was washed with

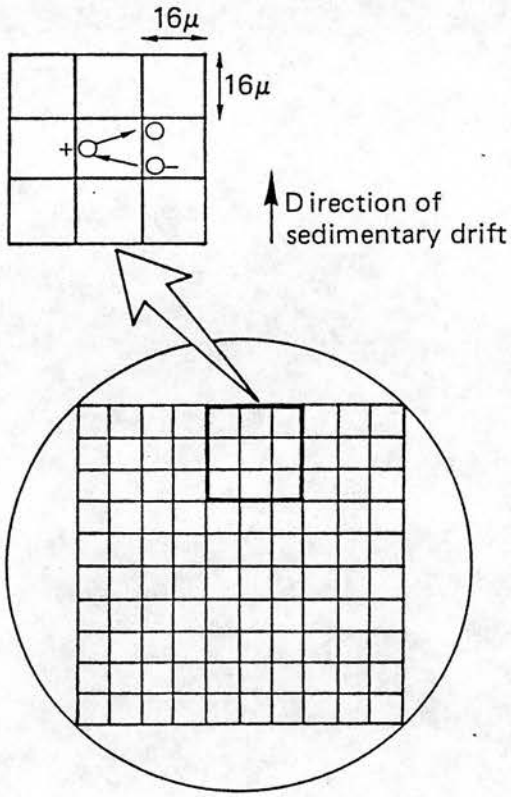


Figure 2.4 The visual field which is seen when looking through the right sided eye piece of the cytopherometer

Hank's BSS before the next sample was introduced.

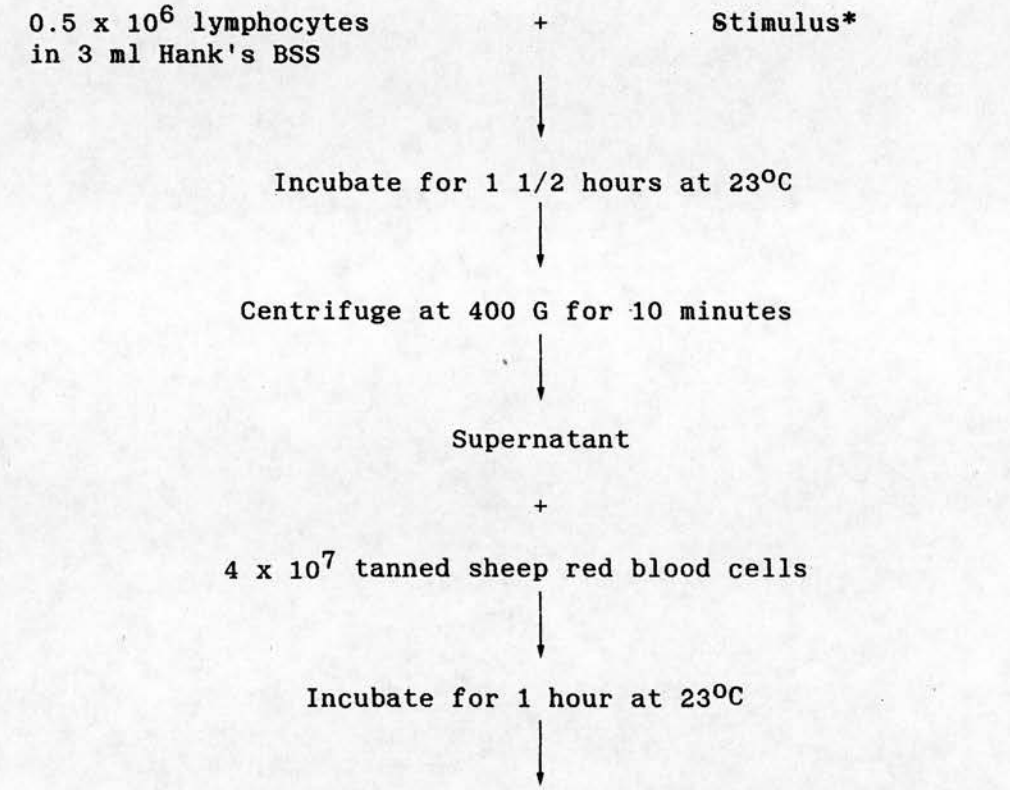
The cytopherometry was performed by Dr. Brian Shenton, Principal Scientific Officer in the Transplantation Laboratory. Dr. Shenton has an international reputation in the field of cytopherometry and has made substantial contributions to the application of cell electrophoresis to clinical medicine. An independent worker in this part of the study enabled the samples to be studied without knowledge of their source and, therefore, complete objectivity was achieved. His willing assistance is gratefully acknowledged.

2.3.3 PERFORMANCE OF THE TEEM TEST

The general method of measuring lymphocyte reactivity using the TEEM test is shown in Figure 2.5. The method has been described previously[327].

2.3.3 (a) Preparation of lymphocytes

Lymphocytes were prepared from PB by the one step sodium metrizoate/Ficoll procedure described by Boyum[345] in 1968. Each blood sample was diluted with an equal volume of Hank's BSS. 10 ml of sodium metrizoate/Ficoll mixture ("Lymphoprep", Nyegaard and Co. Oslo, Norway) was placed in a sterile 20 ml Universal container (Sterilin Ltd., Teddington, Middlesex, England), and 10 ml of diluted blood was layered carefully onto the lymphoprep using a Pasteur pipette. The sterilin container was placed carefully into a centrifuge so that mixing at the blood-lymphoprep interface was avoided. The sample was centrifuged at room temperature for 30 minutes at 1300 revolutions per minute (RPM), resulting in 400 x G at the interface.



* Stimulus (1) PPD : 100 μg
(2) PHA : 10 μg
(3) Con A : 100 μg
(4) PWM : 100 μg

Figure 2.5 The tanned erythrocyte electrophoretic mobility (TEEM) test method

During centrifugation erythrocytes aggregated into rouleaux under the influence of Ficoll and passed to the bottom of the container; lymphocytes formed a layer immediately above the lymphoprep, while leucocytes and platelets remained in suspension in the diluted plasma layer. The lymphocyte layer was removed carefully with a Pasteur pipette and placed in another sterilin container. Lymphocytes were washed twice by adding Hank's BSS and centrifuging at 2000 RPM for 5 minutes at room temperature, and the cell pellet was resuspended in Hank's BSS. Lymphocytes were counted in a Neubauer haemocytometer and the volume adjusted with Hank's BSS so that the final concentration was 1×10^6 lymphocytes per ml.

Using this method of lymphocyte separation, contamination by RBC is minimal and amounts to only a very small percentage of the final cell count[346]. Some immature granulocytes may follow the lymphocytes during separation when there is a granulocyte shift to the left, but patients from whom blood was obtained did not have conditions associated with a granulocyte shift. The main source of contamination of lymphocytes occurs during their removal from the diluted plasma. Lymphocytes appear as a thick white layer immediately above the clear cell-free lymphoprep layer and beneath the pale pink layer of plasma diluted with Hank's BSS. Significant contamination of lymphocytes with diluted plasma was detected by pink discolouration of the lymphocyte suspension and such samples were discarded. The washing procedure removed traces of plasma and platelets from the lymphocyte suspensions.

2.3.3 (b) Lymphocyte-antigen/mitogen interaction

Reactivity of lymphocytes to several stimuli was measured. PPD was used as a stimulating antigen. The assumption was made that the local

population either had been exposed to tuberculosis or had received BCG vaccination. It was accepted locally by clinical microbiologists that in excess of 95% of the regional population had been exposed at some time to tuberculin. PPD was used in the animal studies, but as these in-bred laboratory animals had not been sensitised knowingly to tuberculin, it is likely that PPD acted as a non-specific lymphocyte stimulant (cf mitogen) rather than a recall antigen.

Several mitogens were used. Phytohaemagglutinin (PHA) (Wellcome Reagents Ltd., Beckenham, England) is a lectin derived from the red kidney bean Phaseolus vulgaris and stimulates predominantly T lymphocytes. Concanavalin A (Con A) (Sigma Chemical Company, St. Louis, USA) is a lectin derived from the jack bean and preferentially stimulates suppressor T cells, while PHA preferentially activates helper T cells[347]. Pokeweed mitogen (PWM) (Sigma) is derived from the pokeweed Phytolacca americana and was discovered following observation of cells resembling plasma cells in PB of children who had eaten pokeweed berries. PWM stimulates B lymphocytes and a T cell subset in humans.

2.3.3 (c) Preparation of tanned sheep red blood cells

Tanned sheep red blood cells (TSRBC) are used as indicator particles in the TEEM test and were prepared by a method based on that of Stavitsky[348] and modified by Shenton[349]. Sheep whole blood in Alsevers medium (Appendix 2) (Mercia Diagnostics Ltd., UK) was washed three times in normal saline by adding 1.0 ml packed cells to 20 ml normal saline and centrifuging at 800 G for 10 minutes. Then, 1.0 ml packed washed sheep RBC was diluted with 39 ml of pH 7.2 Stavitsky's phosphate buffered saline (PBS) (Appendix 3) and 1.0 ml of 1 mg/ml tannic acid (Merck, UK) in PBS.

The cell suspension was incubated at 37°C for 10 minutes with constant agitation. Cells were washed three times in PBS and finally resuspended in Hank's balanced salt solution (BSS) (Appendix 4). The TSRBC were kept at 4°C for not more than 24 hours before use.

2.3.3 (d) Expression of results

The supernatant obtained from the interaction of lymphocytes with antigen or mitogen has been shown to decrease the electrophoretic mobility of TSRBC, and the decrease in mobility, or degree of TSRBC slowing, is proportional to the size of the lymphocyte response[327]. In calculating the results of lymphocyte reactivity, the mean time of ten TSRBC migrations over the 32 micron distance for each test sample was compared with the mean migration times of ten TSRBC from two control samples : (1) TSRBC alone, and (2) TSRBC in the presence of antigen or mitogen. The second control was necessary because of the possibility that soluble antigen or mitogens, present in the supernatant which was added to TSRBC, could have bound to the TSRBC and altered their electrophoretic mobility.

The results of antigen and mitogen binding experiments are given in Section 3.1. It was found that antigen and mitogens had no significant effect on TSRBC electrophoretic mobility at concentrations used in this study. Therefore, the results of all samples were calculated using the mean migration times of indicator cells in the absence of antigen or mitogen, (i.e. TSRBC alone, or "control TSRBC") using the formula:

$$\frac{Tt - Tc}{Tc} \times 100$$

Tt = mean migration time in seconds of 10 indicator cells after incubation with supernatant ("test TSRBC")

Tc = mean migration time in seconds of 10 control TSRBC

For example:

Mean control TSRBC (Tc) = 5.981 seconds

Mean test TSRBC (Tt) = 7.057 seconds

TSRBC slowing due to the effect of antigen/mitogen on lymphocytes:

$$= \frac{Tt - Tc}{Tc} \times 100$$

$$= \frac{7.057 - 5.981}{5.981} \times 100$$

$$= \frac{1.076}{5.981} \times 100$$

$$= 17.9\%$$

2.3.4 MEASUREMENT OF SUPPRESSIVE ACTIVITY

Suppressive activity (SA) of a plasma sample or fraction of plasma was quantified by measuring the amount of sample required to suppress a standarised lymphocyte response to PPD. The TEEM test was used for measuring SA but it had to be modified as follows (Figure 2.6):

1. Test solutions were incubated with lymphocytes for 30 minutes at 23°C before addition of PPD.
2. Lymphocytes were either autologous or allogeneic with respect to the test plasma or plasma fraction, and so the SA of a sample using autologous lymphocytes was referred to as "autologous SA", and as "allogeneic SA" if the sample was tested against allogeneic lymphocytes.
3. Only PPD was used as the lymphocyte stimulant.

Three control incubations were required for each test sample: (i) TSRBC alone, and (ii) TSRBC with PPD, as explained in Section 2.3.3(d); and (iii) TSRBC with plasma or a fraction of plasma. It was observed that neither PPD at the concentration of 100 μg in 3 ml nor plasma or plasma

0.5×10^6 autologous or allogeneic lymphocytes
in 3 ml Hank's BSS

+

Plasma (50 μl, 25 μl, 12.5 μl or 6.25 μl etc)

↓

Incubate for 30 minutes at 23°C

+

PPD 100 μg

↓

Incubate for 1 1/2 hours at 23°C

↓

Centifuge at 400 G for 10 minutes

↓

Supernatant

+

4×10^7 TSRBC

↓

Incubate for 1 hour at 23°C

↓

Measure electrophoretic mobility of TSRBC

Figure 2.6 The TEEM test method of measuring suppressive activity of plasma

fraction had any significant effect on the electrophoretic mobility of the TSRBC (results presented in Section 3.1). Therefore, lymphocyte responses were expressed in the same manner as stated earlier, that is, as a percentage slowing of TSRBC.

It was found that plasma and plasma fractions consistently inhibited lymphocyte responses to PPD by varying degrees as revealed by varying degrees of TSRBC slowing. The degree of inhibition caused by plasma or a plasma fraction sample was expressed as "% inhibition" using the formula:

$$\frac{Pa - Ppa}{Pa} \times 100 = \% \text{ inhibition.}$$

Pa = % TSRBC slowing as a result of lymphocyte-antigen interaction

Ppa = % TSRBC slowing as a result of lymphocyte-plasma-antigen interaction.

A method had to be found for quantifying the strength of the suppressive activity contained within a plasma sample. The highest plasma dilution which failed to cause any inhibition of lymphocyte responses could have been used but this method presumed that substances in plasma exerted their inhibitory activity according zero order kinetics, a presumption which may not have been justified. Also, it would have involved considerable work in the laboratory: numerous dilutions of plasma samples would have been required to identify precisely the highest dilution which did not inhibit lymphocyte responses. Alternatively, the lowest dilution causing 100% inhibition could have been used, but the same arguments would have applied.

The method chosen for quantification of SA was as follows: eight dilutions of each test sample were prepared (1/60, 1/120, 1/240, 1/480,

1/960, 1/1920, 1/3840, 1/7680) by adding defined volumes of plasma to 3.0 ml BSS (50 µl, 25 µl, 12.5 µl, 6.25 µl, 3.12 µl, 1.56 µl, 0.78 µl and 0.39 µl respectively). The volumes of plasma fractions added to BSS are given in the respective experimental sections. The % inhibition of lymphocyte reactivity for each plasma dilution was plotted on a graph, with the percentage lymphocyte inhibition on the ordinate and the plasma volume used to make up the dilution on the abscissa (Figure 2.7). The volume of plasma at which 50% inhibition would occur was determined by extrapolation and simply read from the graph; this volume was taken as the SA of samples and was recorded in microlitres of plasma. A small microlitre volume of plasma indicated that the test sample had high SA, as only a small volume was required to suppress the standard lymphocyte reaction to PPD by 50%. Conversely, a large plasma volume indicated that a sample had low SA.

2.3.5 PREPARATION OF PLASMA

Plasma samples were obtained from venous blood which was collected in sterile heparinised tubes and centrifuged at 2000 RPM for 20 minutes. The plasma was removed with a Pasteur pipette and placed in plain sterile tubes. All samples were used fresh and untreated.

2.4 PLASMA LYMPHOCYTOTOXICITY TESTS

All plasma samples were examined for lymphocytotoxic antibodies by the method described by Dewar et al[350]. Lymphocytotoxicity tests were performed in duplicate against allogeneic lymphocytes from three healthy adult donors.

All tests were performed at 23°C using Falcon microtest plates. A 1.0 µl sample of test plasma was placed in the test plate well and 2000 fresh allogeneic lymphocytes in 1.0 ml of Hank's BSS were added. After

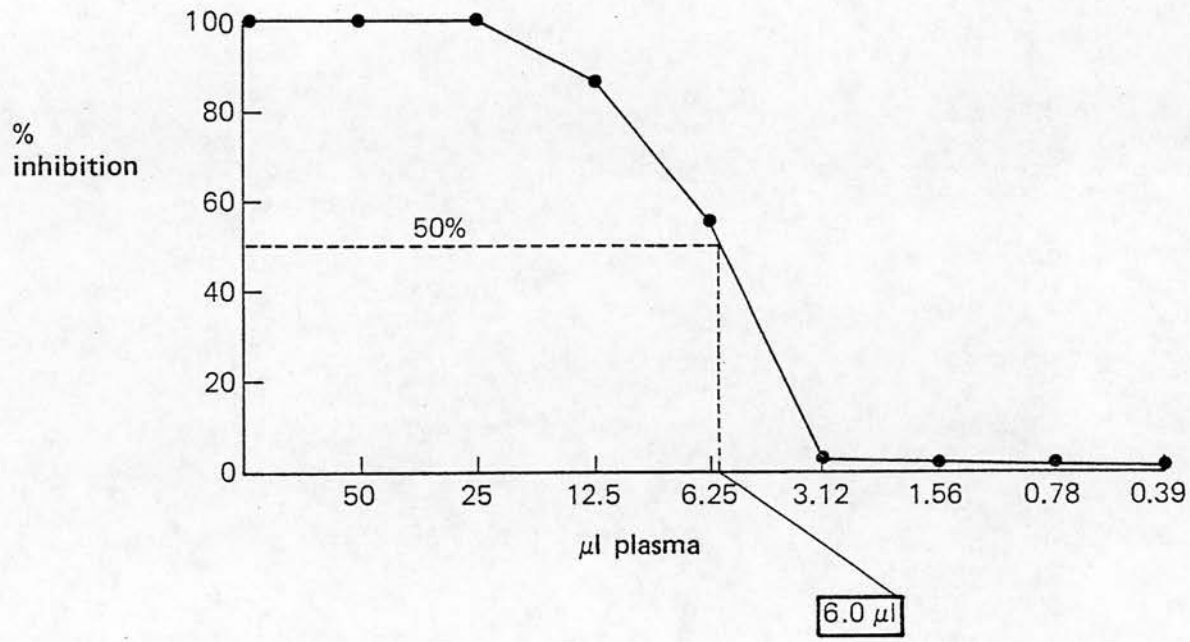


Figure 2.7 The calculation of plasma suppressive activity (PSA)

The percentage inhibition of lymphocyte reactivity of each dilution was plotted graphically; the plasma volume causing 50% inhibition was then read from the graph.

30 minutes 5.0 μl of rabbit complement was added to each well and after incubation for 60 minutes 1.0 μl of 5% Eosin was added. Control wells were set up using the same procedure except that complement was not added. The plates were read immediately under an inverted phase contrast microscope.

A background kill of 5-10% was observed frequently in control plates. A cell kill of 20% or more was regarded as positive. Two plasma samples from chronic renal failure patients who were known to have broadly reacting cytotoxic antibodies to the panel of allogeneic lymphocytes were included as positive controls.

2.5 GEL FILTRATION

Gel filtration column chromatography was used to fractionate plasma samples on the basis of molecular weight. Ultrogel AcA 22 (LKB Produckter AB, Bromma, Sweden) was used as the filtration gel. AcA 22 consists of a polyacrylamide gel (2%) and an agarose gel (2%) matrix with a bead diameter of 60 - 140 microns. The effective fractionation range for globular proteins and peptides is 50,000 - 1,200,000 daltons and so AcA 22 is suitable for fractionating whole plasma.

The chromatography column is shown in Figure 2.8. The column was housed in a cold cabinet at a temperature of 6^{o}C which is within the recommended temperature range for AcA 22 gel.

The main components required for column chromatography are illustrated in Figure 2.9. The buffer reservoir stored Tris-buffer (Appendix 5) which was used for all fractionation studies. Plasma samples were put onto the column by way of a "T" connector in the tubing between the buffer reservoir and the gel column.

A peristaltic pump was situated at the lower end of the column and drew the buffer and plasma sample onto the column at a pre-determined flow

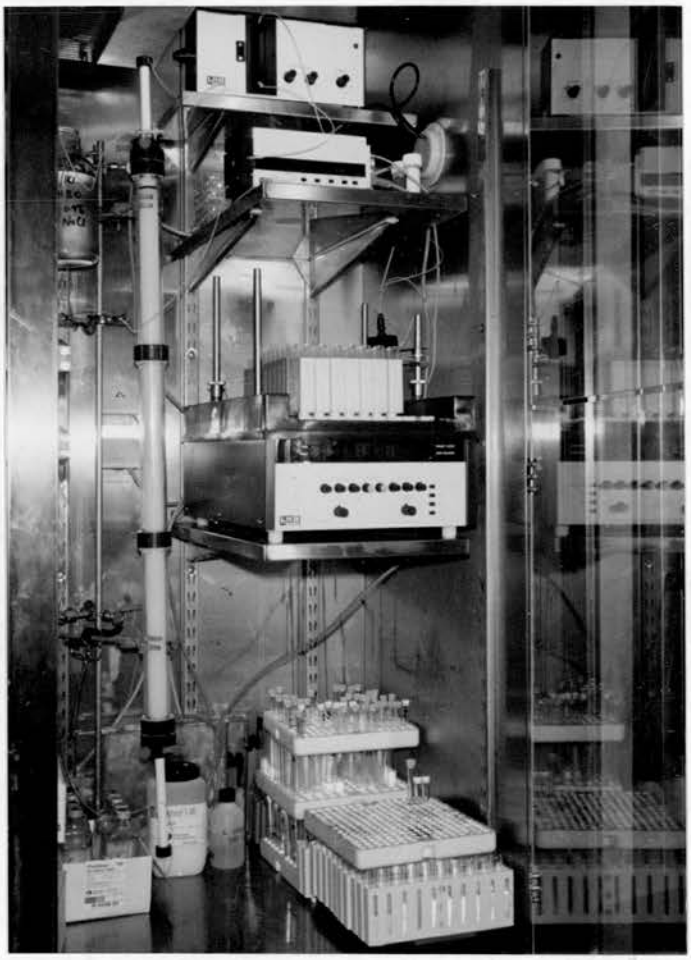


Figure 2.8 The gel filtration chromatography column

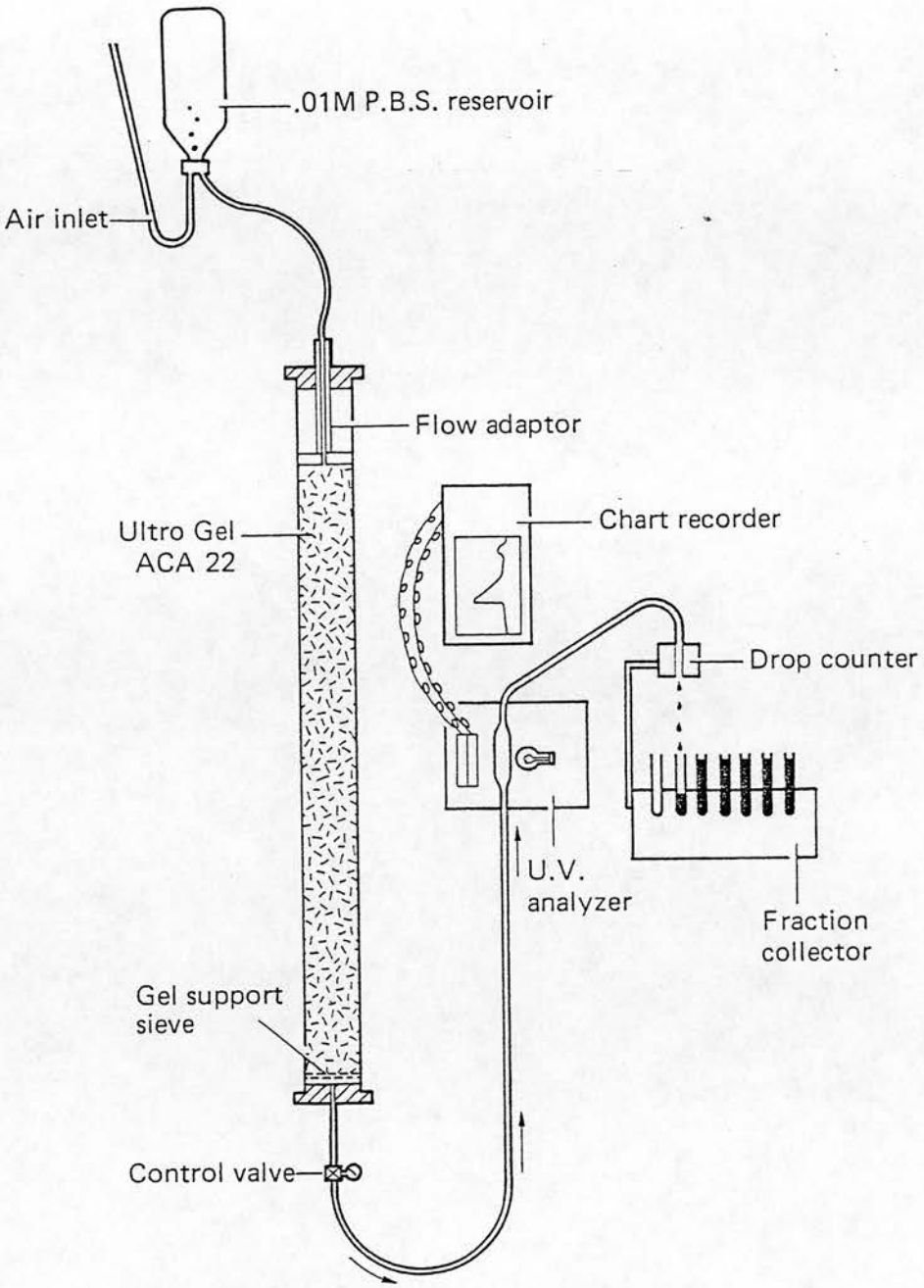


Figure 2.9 A diagram of the gel filtration column

rate. Eluate from the column passed through an ultraviolet analyser (2138 Uvicord S, LKB) which measured the protein concentration spectrophotometrically. The optical density of the eluate at 280 nm was recorded on a chart recorder. The eluate passed to a fraction collector and 100 fractions, each 5.0 ml in volume, were obtained from each plasma sample applied to the column.

2.5.1 PREPARATION OF THE CHROMATOGRAPHY COLUMN

The precision glass tube used for the gel fitration studies was 95 cm in length with an internal diameter of 2.5 cm. Ultrogel AcA 22, which was supplied pre-swollen in citrate buffer with sodium azide, was washed thoroughly in Tris-buffer and dissolved gases were removed by applying a vacuum. The gel was poured along a glass rod into the glass tube so as not to introduce air bubbles into the column of gel. An extension cylinder was attached to the upper end of the glass tube which was then over-filled. During the next two days the gel sedimented so that the gel bed settled into the column beneath the extension cylinder which then contained only buffer. The column was "packed" by running Tris-buffer through it by means of a peristaltic pump. The packing flow rate was 17.0 cm^3/hour and packing was continued for 24 hours. During this equilibration the Uvicord and recorder were started and baseline recordings obtained.

2.5.2 PLASMA FRACTIONATION

The flow rate through the column during fractionation of a plasma sample was 15 cm^3/hour so that three 5.0 ml fractions were obtained each hour. The final length of the gel bed within the glass tube was 82.0 cm. The volume of each plasma sample applied to the gel was 2.0 ml. The void

volume of the column was 110 ml.

Complete elution of a test sample was confirmed by the trace of optical density returning to the base line. Between experiments the pump was switched off and the gel remained in equilibrium with the buffer. If the gel column packed down between experiments, producing a dead space of clear buffer above the gel, a new column was prepared. A summary of details of the gel filtration chromatography column is given in Appendix 6.

2.6 IMMUNO-ELECTROPHORESIS

Immuno-electrophoresis (IE) was used to identify the distribution of proteins within plasma fractions obtained by column fractionation ("fused rocket" technique) and to quantify proteins in plasma and plasma fractions ("single rocket" technique). The method was based on that of Laurell[351].

2.6.1 PREPARATION OF IMMUNO-ELECTROPHORESIS PLATES

2.6.1 (a) Fused rocket immuno-electrophoresis

Glass sheets measuring 205 mm x 110 mm x 2 mm were used as supporting plates for the IE gel. The glass was cleaned meticulously and positioned on a flat adjustable bench top table on which spirit levels were placed. The gel was 1% agarose. One gram of MX Agarose (Mercia Brocades, West Byfleet, Surrey, England) was dissolved in 100 ml Tris-barbitone buffer (TBB) (Appendix 7) by mechanically stirring the suspension while heating to 100^{o}C and then cooling to 60^{o}C.

The required thickness of gel on the glass plate was 2 mm. Therefore, the gel volume was 205 x 110 x 2 = 45.1 mm^{3}. For practical purposes 50 ml of heated agarose solution was prepared for each plate, as a

small amount of gel was lost from adherence to the container in which it was heated.

Antibody to all human plasma proteins ("anti-all") was incorporated into the major part of each IE plate. Some plates had antibodies to IgM, IgG, caeruloplasmin, alpha-1-antitrypsin, transferrin, fibrinogen and alpha-2-macroglobulin (A2M) incorporated into additional bands 2 cm in width, placed between the plain gel band and the main "anti-all" antibody band. Antibodies were used in the following concentrations: anti-all plasma proteins = 4.0 $\mu l/cm^2$ plate area, anti-IgM = 1.0 $\mu l/cm^2$ plate area, anti-IgG = 1.0 $\mu l/cm^2$ plate area, anti-A2M = 1.0 $\mu l/cm^2$ plate area, anti-alpha-1-antitrypsin = 1.5 $\mu l/cm^2$ plate area, anti-albumin = 0.75 $\mu l/cm^2$ plate area, anti-caeruloplasmin = 1.5 $\mu l/cm^2$ plate area, anti-transferrin = 1.0 $\mu l/cm^2$ plate area and anti-fibrinogen = 2.0 $\mu l/cm^2$ plate area.

The lowermost band on the IE plate was the band in which antigen wells were cut and did not contain antibody. A clean straight-edged aluminium bar was placed on the glass plate 25 mm from the lower edge. An appropriate volume (205 x 25 x 2 = 10.25 mm^3) of heated agarose gel was poured onto this part of the plate. The bar was removed after allowing the agarose to cool for 30 minutes, and a 5 mm width of gel was cut away with a scalpel to remove the concave meniscus of gel which formed adjacent to the aluminium bar.

If an agarose band containing mono-specific antibody was to be formed in addition to the main "anti-all" antibody band, the additional band was made next. The aluminium bar was placed on the plate parallel to and 25 mm away from the band of plain agarose. The appropriate volume of heated agarose solution was allowed to cool to 55^oC, antibody was added and mixed evenly into the liquid agarose by mechanical stirring. The agarose

was poured onto the glass plate between the aluminium bar and plain agarose band, and, after the gel had solidified, the bar was removed and the meniscus was trimmed as before.

The "anti-all" band was formed on the remaining plate area. The required volume of gel was prepared, an appropriate amount of antibody was added when the gel reached 55°C, and the liquid agarose was poured as before.

The glass plate was transferred to a hole-cutting frame after the agarose had cooled so that antigen wells could be cut in the plain agarose band. Wells were cut with a sharp-edged steel tube (diameter 2.5 mm) attached to a suction pump. The first row was cut so that the centre of each well was 5 mm from the antibody band. A second staggered row of holes was cut so that the well-centres were midway between the holes of the first row but 5 mm further back from the antibody band. Each well had a volume of 5.0 μl.

2.6.1 (b) Single rocket immuno-electrophoresis

Although minor variations in gel thickness, and hence antibody concentration, are not critical in the determination of protein distribution (fused rocket technique), it is essential that the gel is of uniform thickness for quantification of protein concentrations. Therefore, the agarose plates used for single rocket electrophoresis were prepared in a modified fashion.

A "U"-shaped plastic frame was cut from a single plastic sheet 2 mm thick. It was held firmly with clips between two glass plates (205 mm x 110 mm x 2 mm) so that the frame, once in position, sealed the two short sides and one long side of the space between the two glass sheets

(Figure 2.10). The space between the glass sheets was 185 mm x 100 mm x 2 mm. The volume of gel required was 37 mm^3 and the appropriate amount of antibody was added to the liquid agarose at 55°C. The liquid agarose was introduced into a 50 ml syringe and injected slowly into the space between the two glass plates via an umbilical catheter. When the gel had set, the glass plates were placed horizontally and the clips removed. The upper glass plate was removed carefully, and the lower plate and gel were transferred to the hole-cutting frame. Holes were cut 5 mm apart and 5 mm from a long edge of the plate to form a single row of 36 antigen wells.

2.6.2 PERFORMANCE OF IMMUNO-ELECTROPHORSIS

Each antigen well was filled with a 5.0 μl sample. The agarose IE plate was placed on the cooling plate of the electrophoresis tank (Figure 2.11). The cooling plate consisted of a long hollow coil within a glass slab and cold tap water flowed continuously through the coil. Adjacent to the long sides of the cooling plate was a reservoir containing 500 ml of TBB. Bridges were created between the buffer reservoirs and the long edges of the agarose gel by Whatmann No. 1 filter paper soaked in TBB. Electrodes were inserted into the TBB reservoirs and a current of 20 m amps was applied at a potential difference of 100 volts. The lid was placed securely on the tank to prevent drying of the gel by evaporation, and electrophoresis was performed for 16 hours.

After completion of electrophoresis, the precipitation lines of antigen-antibody complexes within the gel had to be visualised. The surface of each gel was washed with de-ionised water. Four pieces of Whatmann No. 1 filter paper were cut to the same size as the plate and placed on the gel. A flat 2.5 kg weight was applied for 5 minutes. Filter

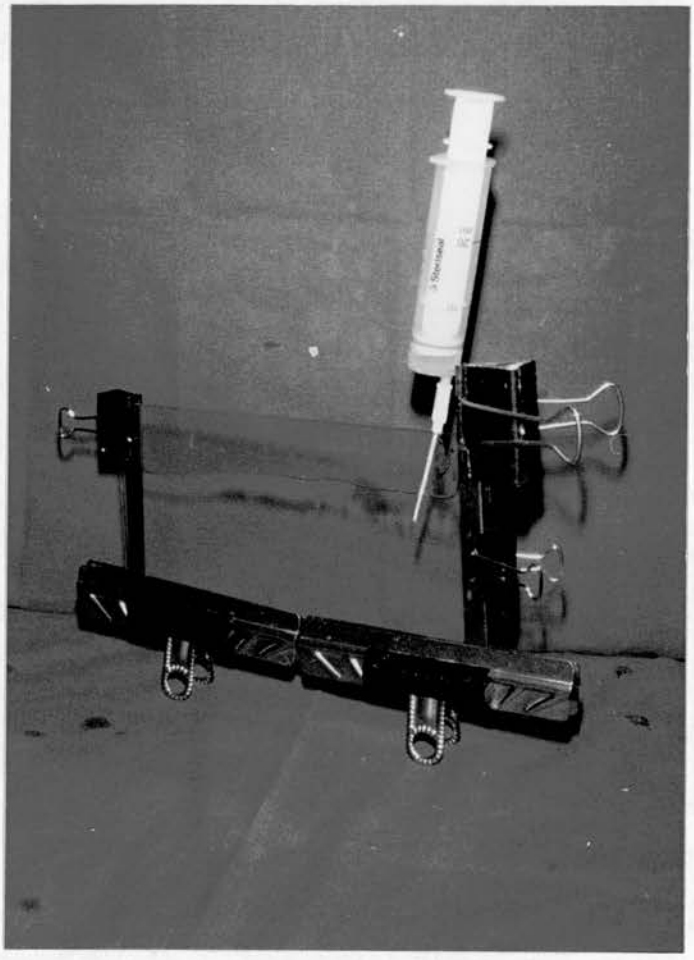


Figure 2.10 The "U" shaped frame used to make the antibody containing gel for single rocket immuno-electrophoresis

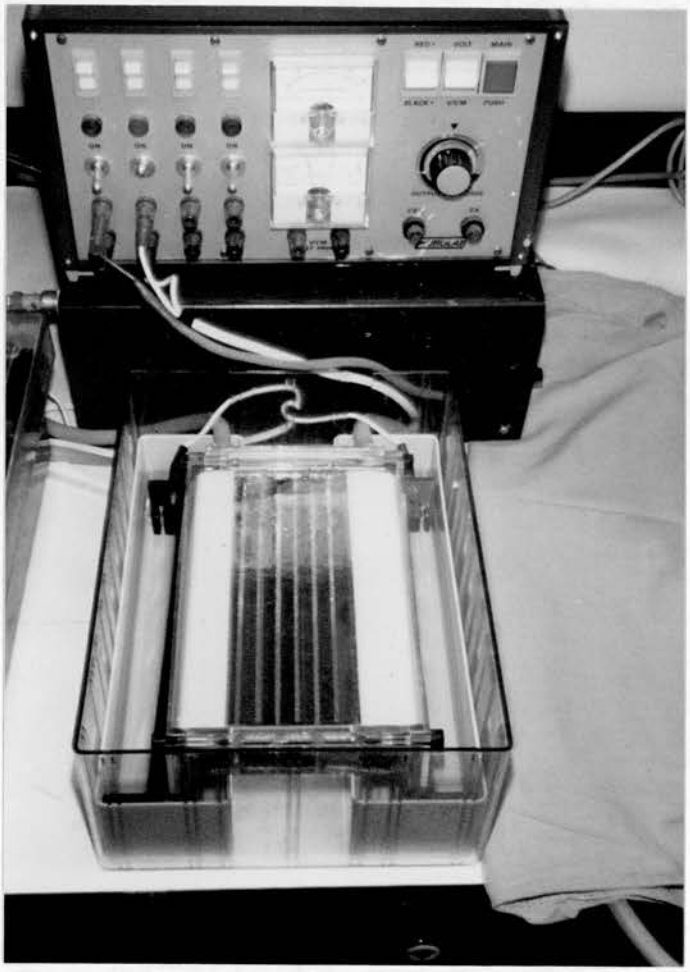


Figure 2.11 The electrophoresis tank in which the immuno-electrophoresis experiments were performed

papers were removed and the gel was washed in 0.1 M NaCl for 15 minutes to remove non-precipitated protein. After pressing again, the gel was washed in de-ionised water for 15 minutes. The plate was pressed for a third time and dried at 30°C until the thin gel layer was firm. It was stained with Coomasie Brilliant Blue solution (Appendix 8) for 15 minutes and then washed immediately in de-staining solution (Appendix 9). The protein precipitates stained dark blue and the background was pale blue (Figure 2.12).

2.6.3 QUANTIFICATION OF SINGLE ROCKETS

Each test sample was prepared in two dilutions and each dilution assayed in duplicate. Serial dilutions of a standard reference protein were applied to antigen wells on the left of each plate. Thus, the height of precipitation rockets of test samples could be compared with those of the reference protein of known concentration. The height of each rocket was taken as the distance from the centre of each antigen well to the tip of the rocket. Dilutions of standard reference proteins and test sera and concentrations of standards are given in Appendix 10.

2.7 IMMUNO-SPECIFIC AFFINITY CHROMATOGRAPHY

Immuno-specific affinity chromatography (IAC) was used to remove A2M, IgG and Fc subunits of IgG from plasma samples and pooled fractions of plasma. Sepharose 4B Gel (Pharmacia Chemicals, Uppsala, Sweden) was used as the IAC column gel. Sepharose 4B consists of spherical 4% agarose gel beads of 40 - 190 μm diameter which have an open pore structure so that the interior of the matrix is available for ligand attachment. The gel exhibits very low non-specific adsorption, an important feature in a technique which depends on specific binding.

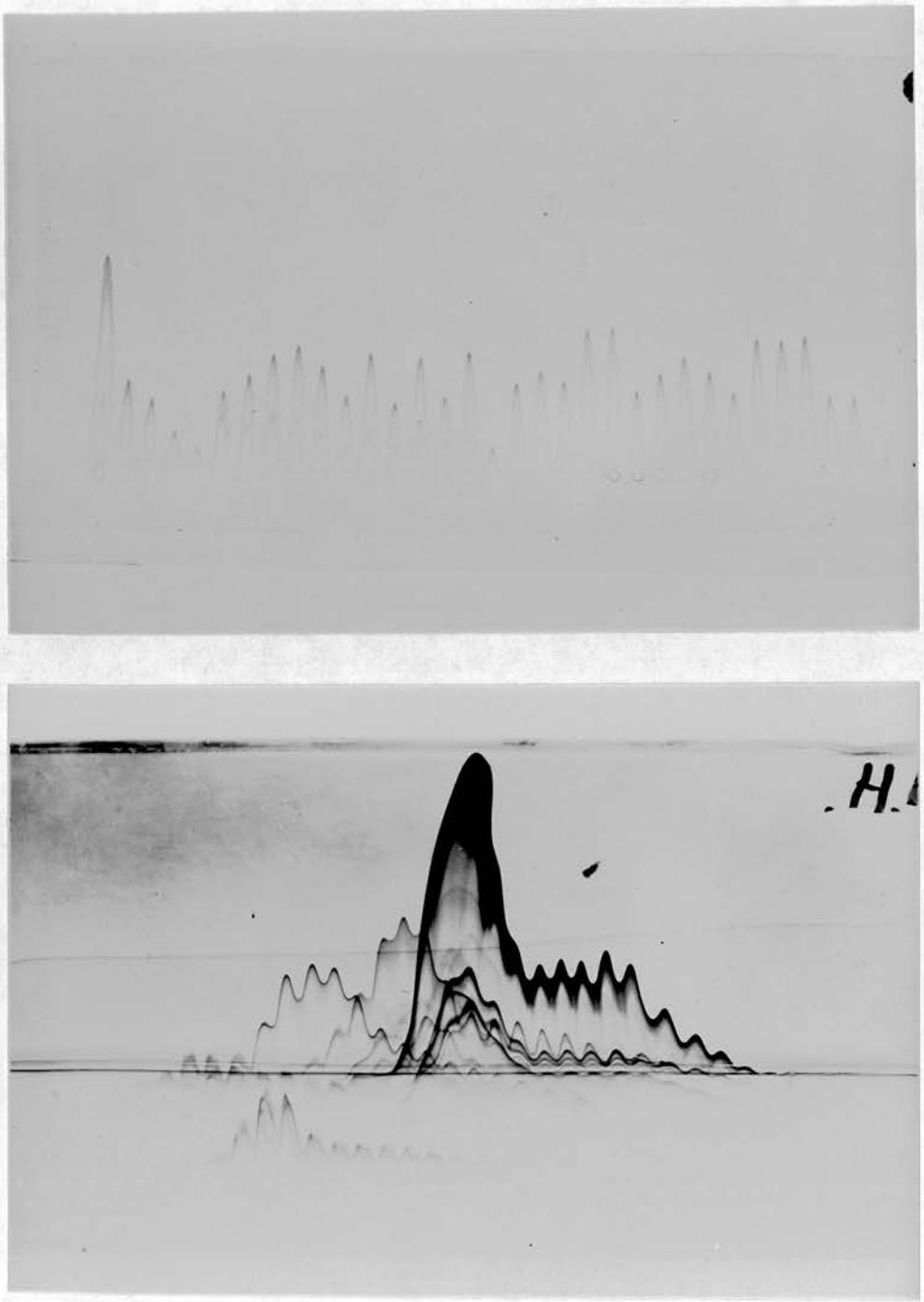


Figure 2.12 Examples of stained immuno-electrophoresis plates produced by single (upper plate) and fused (lower plate) rocket techniques.

2.7.1 PREPARATION OF IMMUNO-SPECIFIC AFFINITY CHROMATOGRAPHY COLUMNS

The 4B gel was washed three times before being coupled with antibody. Three grams of Sepharose beads were placed in a sintered glass funnel attached to a vacuum flask and washed with 100 ml 1 M hydrochloric acid; after 15 minutes a vacuum was applied until the beads were nearly dry. The washing procedure was repeated twice and the beads were washed finally with 100 ml Stavitsky's PBS (Appendix 3).

Antibodies against A2M, IgG and Fc fragments were coupled to the sepharose. Two ml of each antibody were precipitated with 2.5 ml saturated ammonium sulphate at room temperature and the supernatant discarded after centrifugation at 3000 RPM for 10 minutes. The precipitate was dissolved in 10 ml Stavitsky's PBS; ammonium sulphate was removed by three one hour dialyses, against 500 ml Stavitsky's PBS, and the antibody preparation was added to Sepharose beads in sterilin tubes. After gentle mixing on a Matburn rotary mixer at room temperature for two hours, the beads were washed on a sintered glass funnel with 50 ml Stavitsky's PBS to remove excess antibody. The beads were resuspended in 12 ml 1 Molar ethanolamine (pH 8.0) at room temperature for two hours to block any reactive groups remaining after coupling. The beads were washed sequentially in a sintered glass funnel with 50 ml 0.2 M Walpoles acetate buffer (Appendix 11), 50 ml 0.1 M Clark-Lubbs Borate buffer, (Appendix 12), and 50 ml Stavitsky's PBS, to inactivate any active radicals remaining in the beads and to remove non-covalently bound protein. The gel was resuspended in 10 ml of Stavitsky's PBS in a sterile container.

Affinity chromatography columns were made by placing a small pad of glass wool within the barrel of a 10 ml plastic syringe from which the plunger had been removed. A three-way tap was attached to the tip of the barrel, and 5 ml of coupled Sepharose gel was placed into each syringe.

The glass wool supported the gel and prevented it from leaving the syringe whereas buffer could flow through the gel and glass wool pad. When not in use the columns were filled with Stavitsky's PBS with 1% sodium azide, a bacteriostatic agent, and a cap placed over the open end of each column. Columns were stored at 6°C.

A control column was made using normal rabbit serum, instead of antibody, coupled to the Sepharose beads.

2.7.2 PERFORMANCE OF IMMUNO-SPECIFIC AFFINITY CHROMATOGRAPHY

Each column was washed with 20 ml Stavitsky's PBS before use. Test samples were applied to the top of the column, the amount of each sample being either 0.5 ml plasma or the volume of a plasma fraction which contained the equivalent of 0.5 ml plasma. Each test sample was incubated in a column at room temperature for 30 minutes and then eluted with 20 ml Stavitsky's PBS and collected.

Adsorbed protein was removed from the Sepharose column by washing with 20 ml of 3 Molar sodium thiocyanate. Sodium thiocyanate was removed from the eluate by dialysing against 500 ml Hank's BSS for four hours. The column was washed with 20 ml Stavitsky's PBS after use, and the adsorbed protein was collected.

2.8 DETECTION OF IMMUNE COMPLEXES

Immune complexes (IC) were detected in pooled fractions of plasma by precipitation with polyethylene glycol (PEG), an uncharged, synthetic, linear polysaccharide. When added to serum, PEG precipitates proteins in proportion to its concentration and the molecular size of the proteins. For example, 20% PEG precipitates most native immunoglobulins and many

other proteins; 5% PEG precipitates a significantly smaller proportion of immunoglobulins but the same proportion of IC[352]. The PEG method of IC precipitation is well accepted, straight-forward, reproducible and correlates well with other methods of IC detection[353,354].

A criticism of the PEG method is that free and non-specifically aggregated immunoglobulins may be precipitated and wrongly interpreted as true IC. Therefore, steps were taken to eliminate this source of error by not testing whole plasma or serum but rather plasma fractions after removal of IgM and IgG by gel filtration. Also, procedures which encourage non-specific antigen-antibody aggregation, such heating or freeze-thawing of samples, were avoided.

The method of PEG precipitation of IC was that of Creighton[352]. Solutions of 5%, 10% and 20% PEG of MW 6000 (BDH Chemicals Ltd, Poole, England) were made with 0.1 Molar borate buffer (Appendix 12). Each test sample consisted of only the heaviest fractions of plasma obtained from gel filtration, these fractions having been pooled to form a single region or peak ("region 1") for each plasma sample. (The method of pooling gel filtration fractions is explained in Section 3.6.2). The volume of each test sample was 5% of the total volume of each region 1, and was added to an equal volume of each PEG solution. Test samples were prepared in duplicate, resulting in 6 samples (5% x 2, 10% x 2, 20% x 2) for the region 1 of each subject's plasma specimen.

Test samples were incubated with PEG for 18 hours at $4^{o}C$, and then centrifuged at 5000 G for 20 minutes. Each precipitate appeared as a white pellet at the bottom of the conical centrifuge tube. The supernatant was aspirated and collected, and the PEG removed from the supernatant by ultrafiltration, using an ultrafiltration membrane with an exclusion limit of 300,000 daltons. The supernatant was tested for SA after removing PEG.

The precipitate was dissolved in 3.0 ml Hank's BSS by vigorous shaking and then tested for SA.

2.9 RADIAL IMMUNODIFFUSION

Single radial immunodiffusion (RI) was used to measure IgM, IgG and albumin concentrations in samples of plasma and fractions of plasma. RI plates containing antibody to IgM, IgG - Fc or albumin and appropriate standard antigens were purchased (Behringwerke AG, Marburg, W. Germany).

Test samples were prepared in two dilutions, each of 5.0 μl volume. RI plates were placed on a flat adjustable table within a humid, sealed plastic box. Spirit levels were used to check that the table was level. Antigens (test samples or standards of known concentration) were added to antigen wells and the plates were left undisturbed for 3 days. The diameters of the resulting precipitation rings were measured. Standard curves were constructed by plotting the squares of diameters of the precipitation rings for each standard against their concentrations[355], and the values of the test samples were read off from the graphs.

2.10 STATISTICAL METHODS

Statistical analysis of results was performed with parametric and non-parametric tests. The Students t test, a parametric statistical test formulated largely by Gosset in 1908, was used whenever the data were distributed normally, were independent of each other and had similar degrees of variance. The non-parametric statistical tests were the Mann-Whitney U test[356] and the Wilcoxon two sample rank sum test for unpaired data[357]. They were used when the above criteria for use of a parametric test could not be fulfilled.

Correlations between variables were investigated by constructing scatter diagrams and calculating the correlation coefficient r. Scatter diagrams were drawn by plotting the dependent variable against the vertical (y) axis and the independent variable against the horizontal (x) axis for each pair of data to be correlated. The coefficient of correlation (r) was calculated from the formula:

$$r = \frac{\sum (x_1 - x)\,(y_1 - y)}{\sqrt{\sum (x_1 - x)^2 \sum (y_1 - y)^2}}$$

where

x_1 = values of the independent variable

x = the mean of the x observations

y_1 = values of the dependent variable

y = the mean of the y observations

The significance of correlation was determined by calculating the value of t from the formula :

$$t = r \sqrt{\frac{n - 2}{1 - r^2}}$$

where

r = correlation coefficient

n = number of observations

The test of significance was considered to be valid if the data points of the scatter diagrams had an approximate elliptical shape, indicating normal distribution of the two variables[358].

CHAPTER 3

HUMAN EXPERIMENTAL STUDIES

3.1 STUDIES OF THE EFFECTS OF ANTIGEN AND MITOGEN ON TANNED SHEEP ERYTHROCYTE ELECTROPHORETIC MOBILITY

The results of a series of experiments aimed at defining the effects of PPD antigen and PHA, Con A and PWM mitogens on the electrophoretic mobility of TSRBC are reported in this section. These experiments had to be performed so that any influence of the lymphocyte stimulating antigen or mitogen could be taken into account when measuring lymphocyte reactivity (Section 2.3.3.(d)).

3.1.1 METHOD

The electrophoretic mobility of TSRBC in Hank's BSS was measured, as described in Section 2.3.3. Seven concentrations of PPD, PHA, Con A and PWM were made up in 3.0 ml of Hank's BSS containing 4×10^7 TSRBC. The concentrations were: 10 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, 600 μg/ml, 800 μg/ml, 1000 μg/ml. TSRBC were incubated with antigen or mitogen for one hour at 23°C and then the electrophoretic mobility of ten TSRBC from each test incubation was measured. Results were expressed as % slowing of TSRBC. The mean electrophoretic mobility (in seconds) of ten TSRBC from each test incubation was compared with that of ten TSRBC alone. This latter value was taken as the "zero" point: therefore, a positive result indicated inhibition of TSRBC by antigen or mitogen while a negative value indicated an increase in electrophoretic mobility compared with untreated TSRBC. The Student's t test was used for statistical analysis.

3.1.2 RESULTS

The results are given in Tables 3.1 - 3.4 and illustrated in Figure 3.1. PPD and PWM caused only minimal slowing of TSRBC; inhibition tended to increase with increasing antigen/mitogen concentration. However,

Table 3.1 The effect of PPD on the electrophoretic mobility of tanned sheep red blood cells (TSRBC)

Dose of PPD (μl/ml)	% slowing of TSRBC (mean ± 1SD, n = 10)	p value - PPD x Control
10	0.3% ± 1.1%	NS
100	0.6% ± 1.2%	NS
200	1.1% ± 1.4%	NS
400	0.9% ± 1.3%	NS
600	1.4% ± 2.7%	NS
800	3.8% ± 1.4%	<0.001
1000	2.0% ± 1.5%	<0.01

Table 3.2 The effect of PHA mitogen on the electrophoretic mobility of tanned sheep red blood cells (TSRBC)

Dose of PHA (μl/ml)	% slowing of TSRBC (mean ± 1SD, n = 10)	p value - PHA x Control
10	0.4% ± 1.3%	NS
100	1.1% ± 0.8%	<0.01
200	3.2% ± 0.9%	<0.001
400	18.9% ± 1.4%	<0.001
600	19.5% ± 1.1%	<0.001
800	28.8% ± 7.9%	<0.001
1000	36.8% ± 1.9%	<0.001

Table 3.3 The effect of Con A mitogen on the electrophoretic mobility of tanned sheep red blood cells (TSRBC)

Dose of Con A (μl/ml)	% slowing of TSRBC (mean ± 1SD, n = 10)	p value - Con A x Control
10	1.0% ± 0.5%	NS
100	1.4% ± 0.8%	NS
200	0.9% ± 0.6%	NS
400	1.3% ± 1.6%	NS
600	4.2% ± 2.5%	<0.01
800	7.9% ± 2.4%	<0.001
1000	9.4% ± 1.0%	<0.001

Table 3.4 The effect of PWM mitogen on the electrophoretic mobility of tanned sheep red blood cells (TSRBC)

Dose of PWM (μl/ml)	% slowing of TSRBC (mean ± 1SD, n = 10)	p value - PWM x Control
10	0.5% ± 2.3%	NS
100	0.8% ± 1.2%	NS
200	2.1% ± 2.9%	NS
400	1.8% ± 1.3%	NS
600	1.2% ± 0.8%	NS
800	1.1% ± 0.9%	NS
1000	3.4% ± 3.6%	<0.05

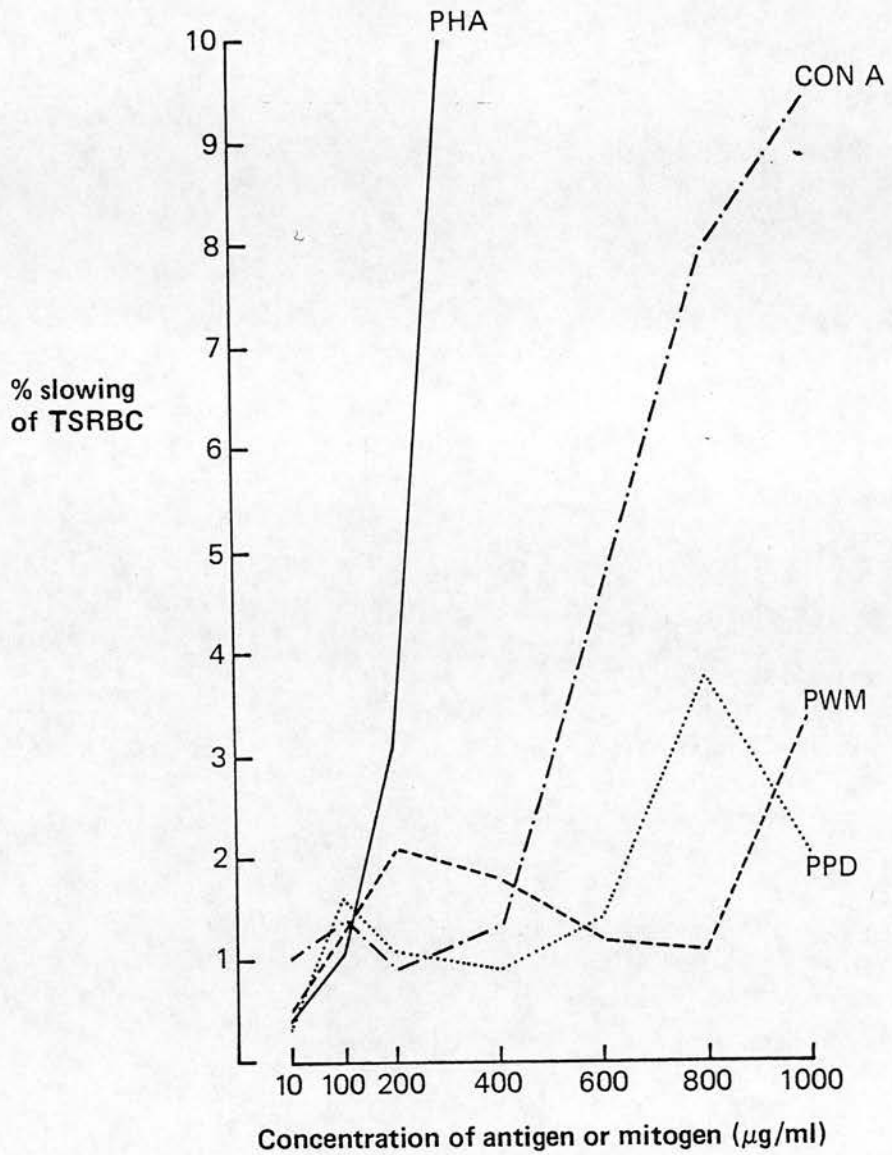


Figure 3.1 The effect of antigen (PPD) or mitogen (PHA, Con A or PWM) on the electrophoretic mobility of tanned sheep red blood cells (TSRBC)

only at concentrations of 800 μg/ml (PPD) and 1000 μg/ml (PPD and PWM) did TSRBC mobilities differ significantly from control values. PHA and Con A caused significant interference with TSRBC electrophoretic mobility at much lower concentrations: the only concentration of PHA which did not cause significant slowing was 10 μg PHA/ml, and concentrations of Con A greater than 400 μg/ml inhibited TSRBC mobility significantly.

3.1.3 DISCUSSION

The experiments demonstrate that PPD, PHA, PWM and Con A can alter the electrophoretic mobility of TSRBC although, with the exception of PHA, alteration of surface electric charge occurred only at relatively high concentrations. The antigen and mitogens did not cause TSRBC clumping or aggregation even at high concentrations. Obviously, it was necessary to use these lymphocyte stimulants at relatively low concentrations in subsequent experiments so that they did not influence TSRBC electrophoretic mobility. The concentrations used throughout this study were:

PPD : 100 μg per 3 ml test sample - 33 μg/ml

PHA : 10 μg per 3 ml test sample - 3.3 μg/ml

PWM : 100 μg per 3 ml test sample - 33 μg/ml

Con A : 100 μg per 3 ml test sample - 33 μg/ml

These concentrations of each stimulant were within the ranges that had no significant effect on TSRBC electrophoretic mobility, so that any alteration in TSRBC mobility detected in subsequent experiments could not be ascribed to a direct effect of antigen or mitogen interfering with the indicator cells.

3.2 STUDIES OF LYMPHOCYTE REACTIVITY

3.2.1 INFLUENCE OF DISEASE

As a prerequisite to the study of plasma factors which may influence lymphocyte reactivity, it was necessary to measure lymphocyte reactivity in an environment free of plasma so that a baseline value of lymphocyte reactivity could be established. Thus, *in vivo* tests of lymphocyte function could not be used. However, it is accepted generally that *in vitro* reactivity of lymphocytes to antigenic stimulation is a correlate of cell-mediated immunity[347] and so an *in vitro* test (the TEEM test) was used to measure lymphocyte reactivity in the absence of plasma.

3.2.1 (a) Method

Lymphocytes were obtained from 136 subjects by the method described in Section 2.3.3(a). The experimental subjects have been described in Section 2.1 and further details are given in Table 3.5. Lymphocytes were stimulated with PPD, PHA, Con A and PWM. The TEEM test method has been described in Section 2.3. The Student's t test was used for statistical analysis of results.

3.2.1 (b) Results

Lymphocyte responses to PPD, PHA, Con A and PWM are shown in Figures 3.2 - 3.5 respectively.

Responses to PPD were uniform in normal subjects, with a mean of 19.2% TSRBC slowing. Responses of patients with benign diseases had a wider distribution which was skewed towards lower values. Their responses were significantly lower than those of the normal group ($p<0.001$). The responses of the malignant group were distributed widely also and were significantly lower than those of the benign group ($p<0.001$). Similar

Table 3.5 Subject groups investigated in the studies of lymphocyte reactivity and plasma suppressive activity

	GROUP		
	Normal	Benign	Malignant
Number of subjects	15	52	69
Age (years) (mean ± 1SD)	45.4 ±10.5	59.3 ±14.1	62.8 ±12.5
Age range (years)	26 - 66	26 - 88	27 - 89
Sex (M : F)	10 : 5	25 : 27	30 : 39

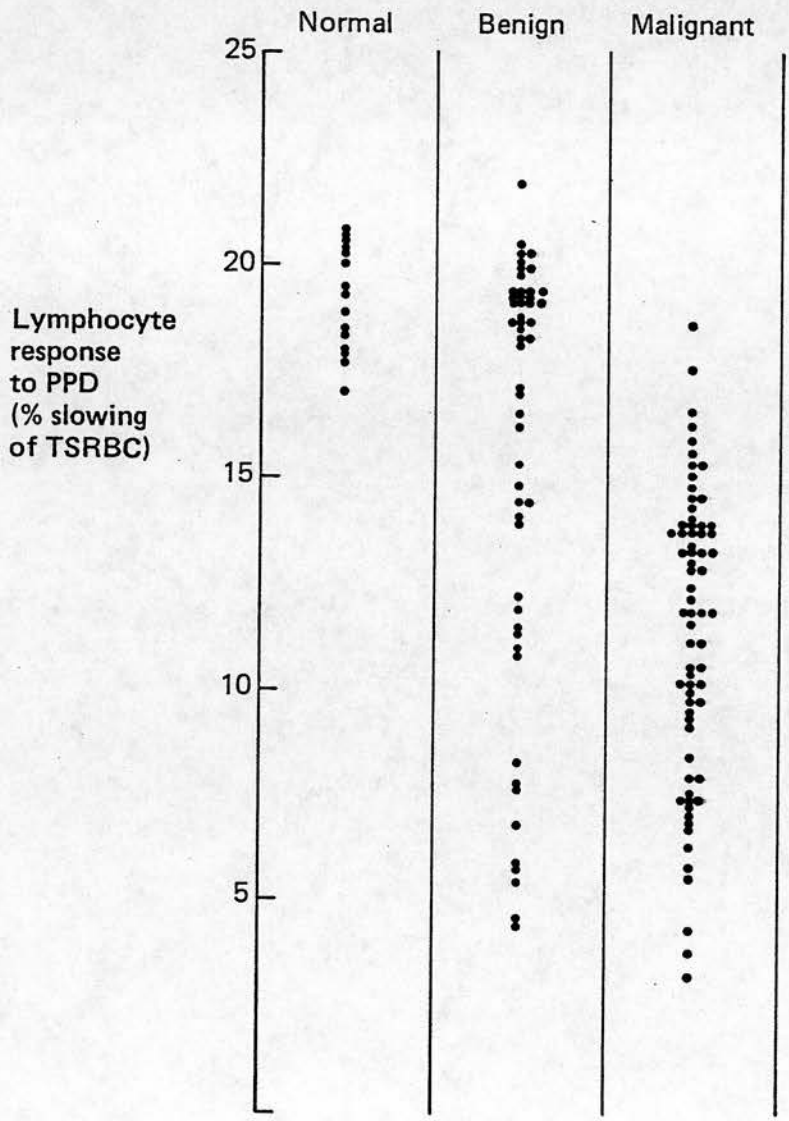


Figure 3.2 Results of <u>in vitro</u> lymphocyte responses to PPD

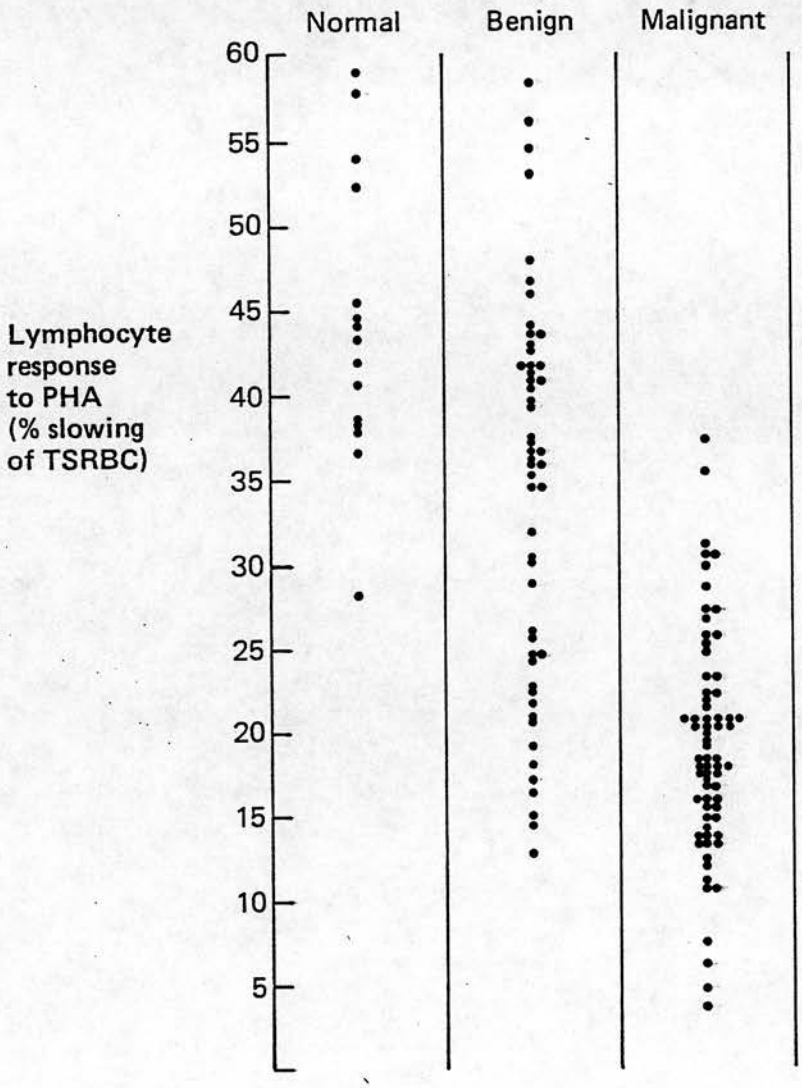


Figure 3.3 Results of <u>in vitro</u> lymphocyte responses to PHA

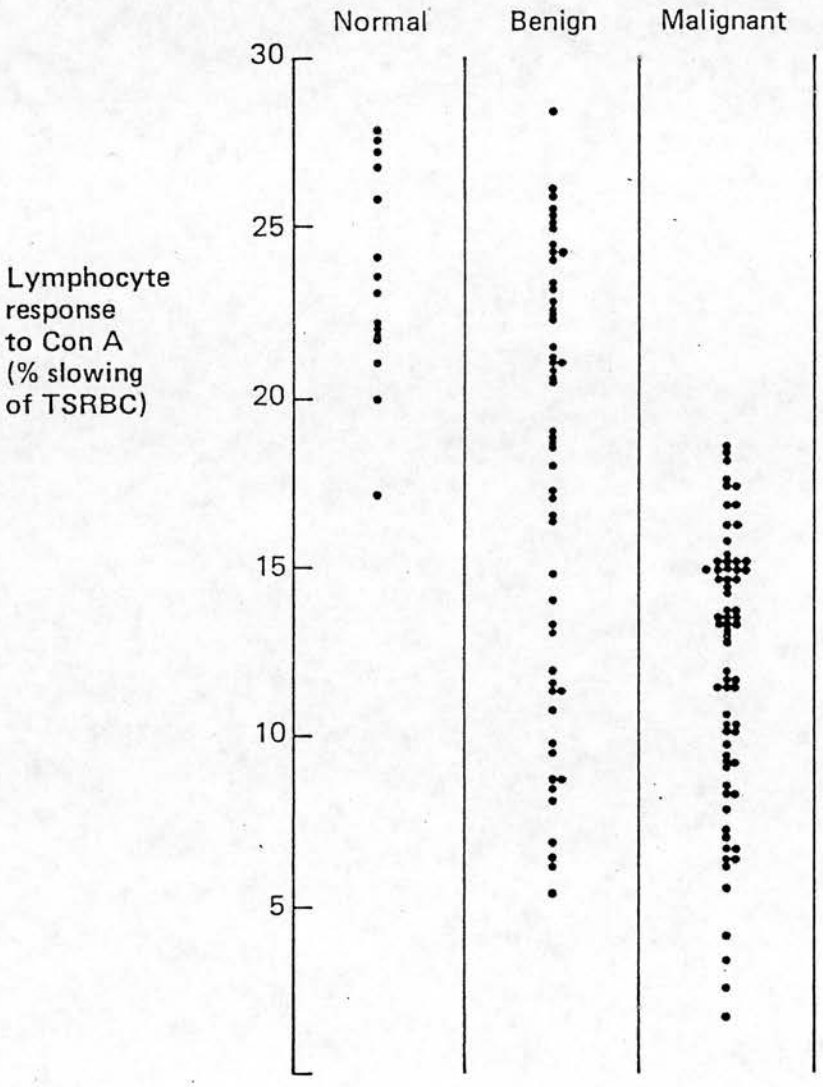


Figure 3.4 Results of <u>in vitro</u> lymphocyte responses to Con A

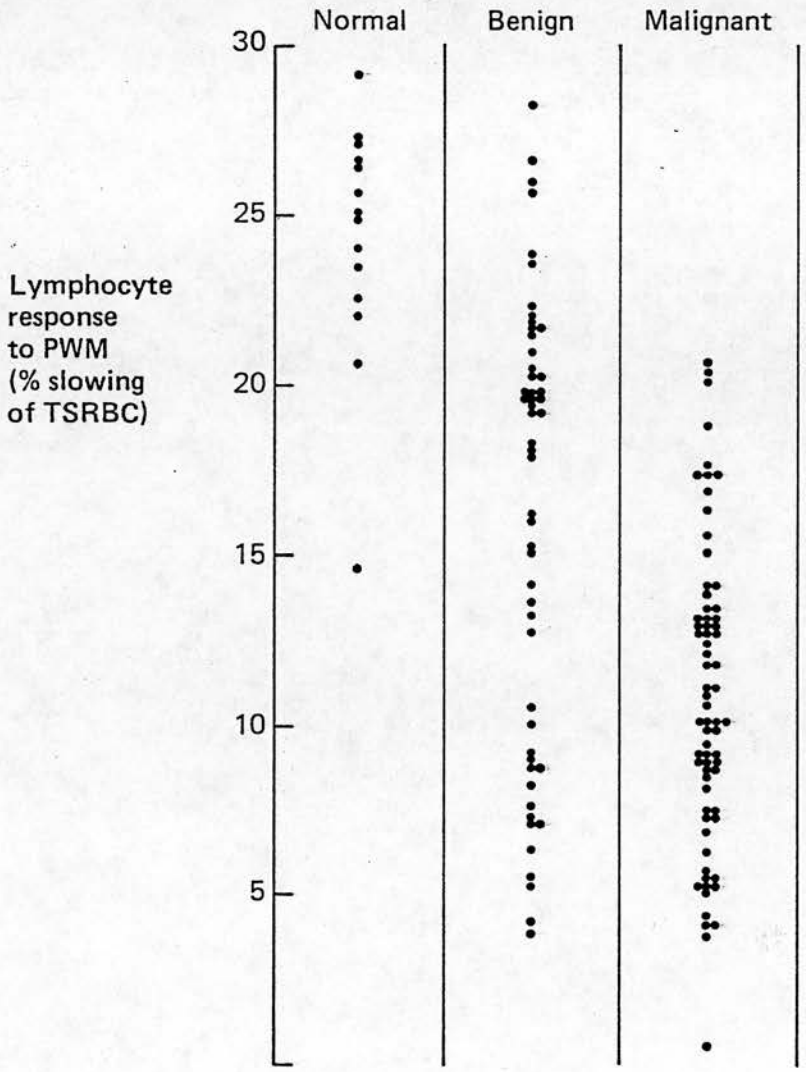


Figure 3.5 Results of <u>in vitro</u> lymphocyte responses to PWM

results were obtained from lymphocyte responses to the three mitogens. Although there was overlap between responses of each group, those of the normal group were significantly greater than those of the benign group, which were greater than responses of the malignant group.

3.2.1 (c) Discussion

The results show that the presence of malignant disease is associated with reduced lymphocyte reactivity as measured by the TEEM test. There was minimal overlap of results from the malignant and normal groups. The benign group had a wide scatter of results, the majority of which fell within the same range as the results of the normal group. This suggested that the benign group contained several subpopulations. Overall, the results suggest that factors in addition to malignancy can influence lymphocyte reactivity.

The normal subjects were not well matched with the other two groups with respect to age or sex distribution. Difficulty was experienced in obtaining blood samples from elderly healthy adults; those admitted to hospital could not be included in the normal healthy group. However, as further analysis did not show any trend or significant correlation between lymphocyte responsiveness and age or sex for any subject group, use of this control group appeared to be valid. Further analyses were undertaken to identify factors other than disease which could explain differences between the groups.

3.2.2 INFLUENCE OF NUTRITIONAL STATUS

As malnutrition has a profound effect on the immune system (Section 1.6.1), the results of lymphocyte reactivity reported in Section 3.2.1 were analysed on the basis of nutritional status.

3.2.2 (a) Method

Each experimental subject underwent nutritional assessment (weight-for-height, weight loss (only in patients), arm muscle circumference (AMC), triceps skin fold thickness (TSF) and serum albumin). Lymphocyte responses to each stimulant were plotted graphically against each nutritional parameter by constructing scatter diagrams. A correlation co-efficient (r) was calculated for each set of data and the t test was used to assess the significance of correlation (Section 2.10).

3.2.2 (b) Results

The co-efficients of correlation (r) and probability values (p) for correlations between lymphocyte responses and nutritional parameters are given in Tables 3.6 and 3.7 for the benign and malignant groups respectively. Scatter diagrams showing lymphocyte responses to PHA plotted against each nutritional parameter are reproduced in Figures 3.6 - 3.10 and 3.11 - 3.15 for the benign and malignant groups respectively. Scatter diagrams for each of the three mitogens have not been included because of limitations of space.

No correlation was found between lymphocyte responses and nutritional parameters in normal subjects.

Lymphocyte responses to each stimulus showed significant correlations with each nutritional parameter in the benign group (Table 3.6). The best correlation was obtained between lymphocyte responses to PHA and the TSF thickness ($r = 0.79$, $p<0.001$). Although the correlation was reasonably strong, the scatter of results was wide, indicating poor sensitivity of lymphocyte responsiveness as an index of body fat stores. In the same way, lymphocyte responses to PPD and the mitogens could be correlated with nutritional parameters but the

Table 3.6 Correlations of lymphocyte responses with nutritional parameters in patients with benign diseases

		COEFFICIENT OF CORRELATION (r)			
NUTRITIONAL PARAMETER	n	PPD	PHA	Con A	PWM
Weight for height	52	0.48	0.53	0.51	0.57
Weight loss	25	-0.66	-0.78	-0.72	-0.78
Triceps skin fold	52	0.73	0.79	0.79	0.76
Arm muscle circumference	52	0.68	0.66	0.59	0.60
Serum albumin	52	0.54	0.61	0.76	0.72

All correlations were significant at $p < 0.001$

Table 3.7 Correlations of lymphocyte responses with nutritional parameters in patients with malignant diseases

		COEFFICIENT OF CORRELATION (r)			
NUTRITIONAL PARAMETER	n	PPD	PHA	Con A	PWM
Weight for height	69	0.35#	0.32"	0.27"	0.15+
Weight loss	55	-0.55#	-0.43#	-0.54#	-0.31"
Triceps skin fold	69	0.31"	0.53#	0.19+	0.10+
Arm muscle circumference	69	0.49#	0.44"	0.17+	0.10+
Serum albumin	69	0.39#	0.25*	0.37*	0.19+

Values of p : + = Not significant

* = $p < 0.05$

" = $p < 0.01$

\# = $p < 0.001$

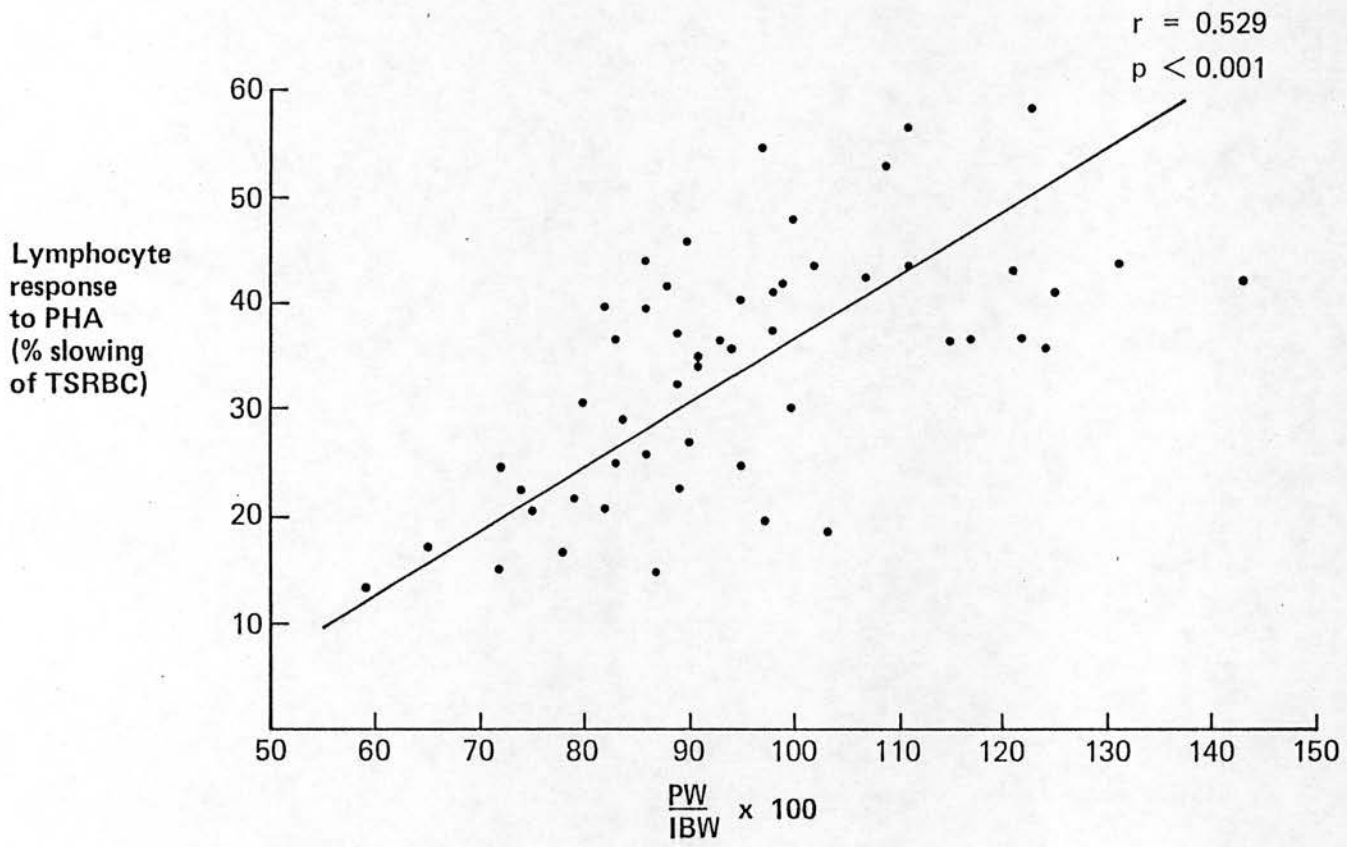


Figure 3.6 Correlation of lymphocyte responses to PHA with percentage weight for height in patients with benign disease

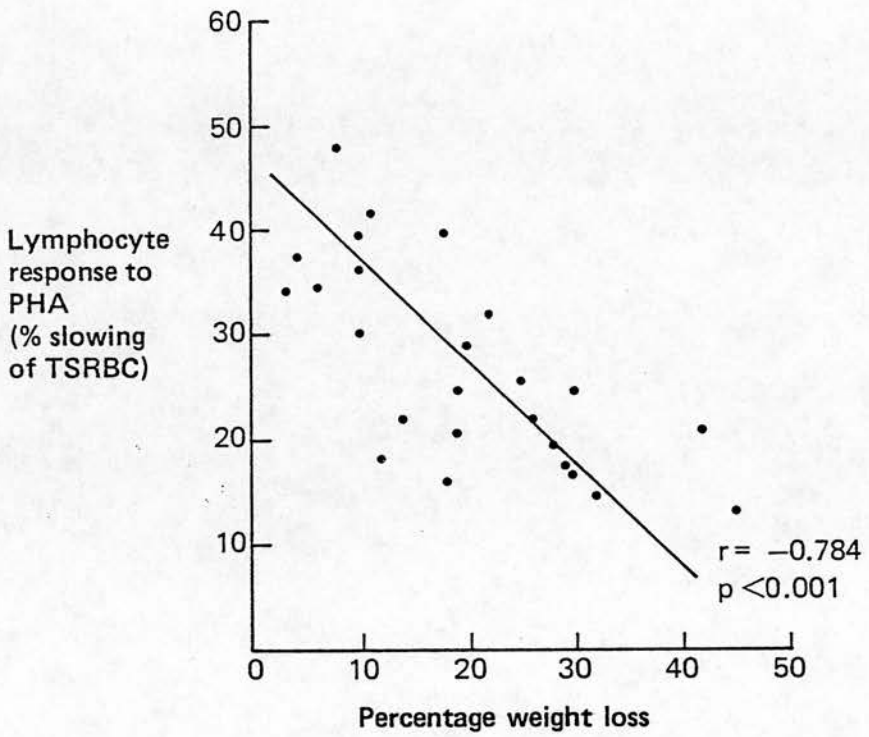


Figure 3.7 Correlation of lymphocyte responses to PHA with percentage weight loss in patients with benign disease

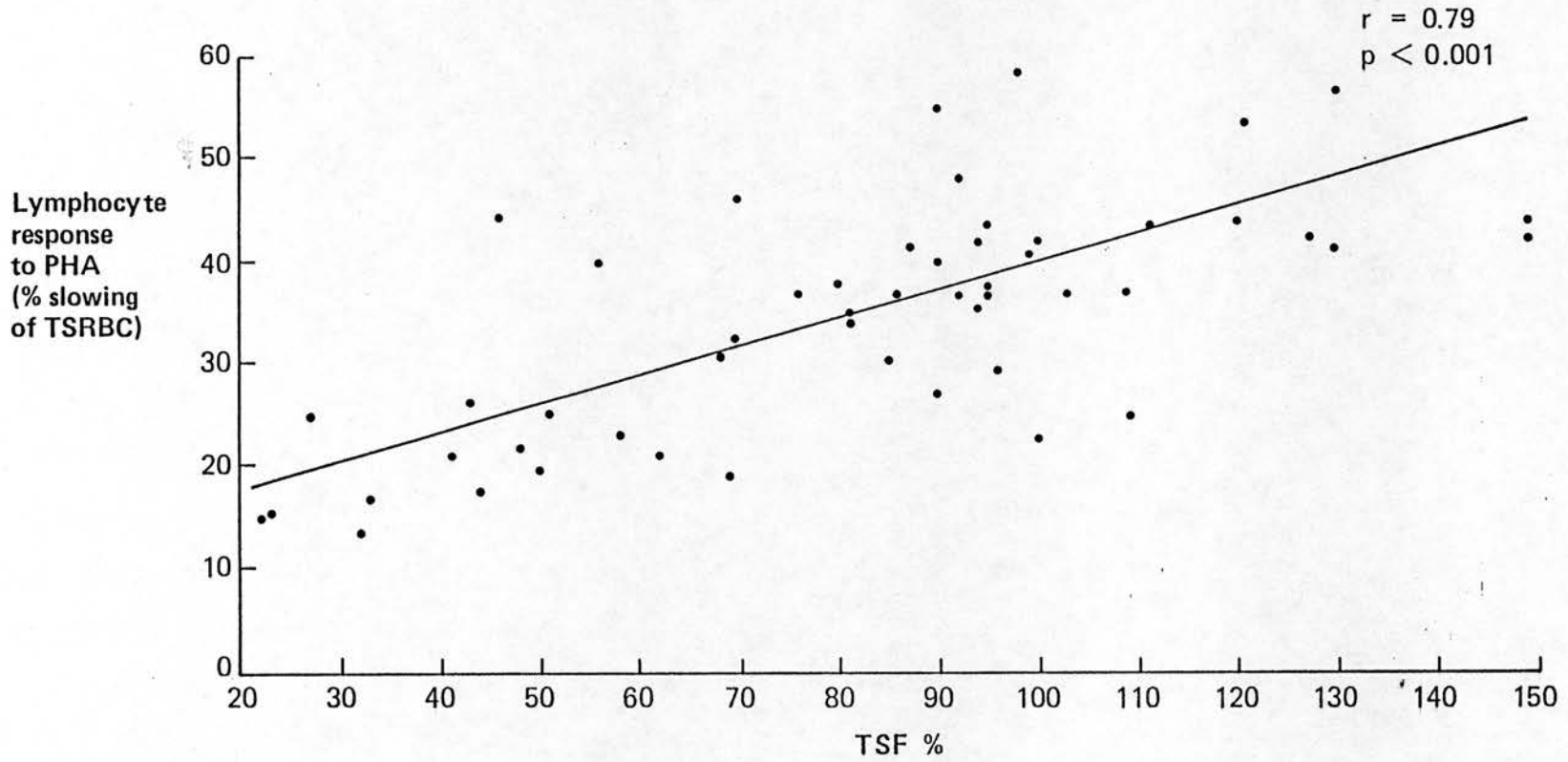


Figure 3.8 Correlation of lymphocyte responses to PHA with triceps skin fold (TSF) thickness as a percentage of normal in patients with benign disease

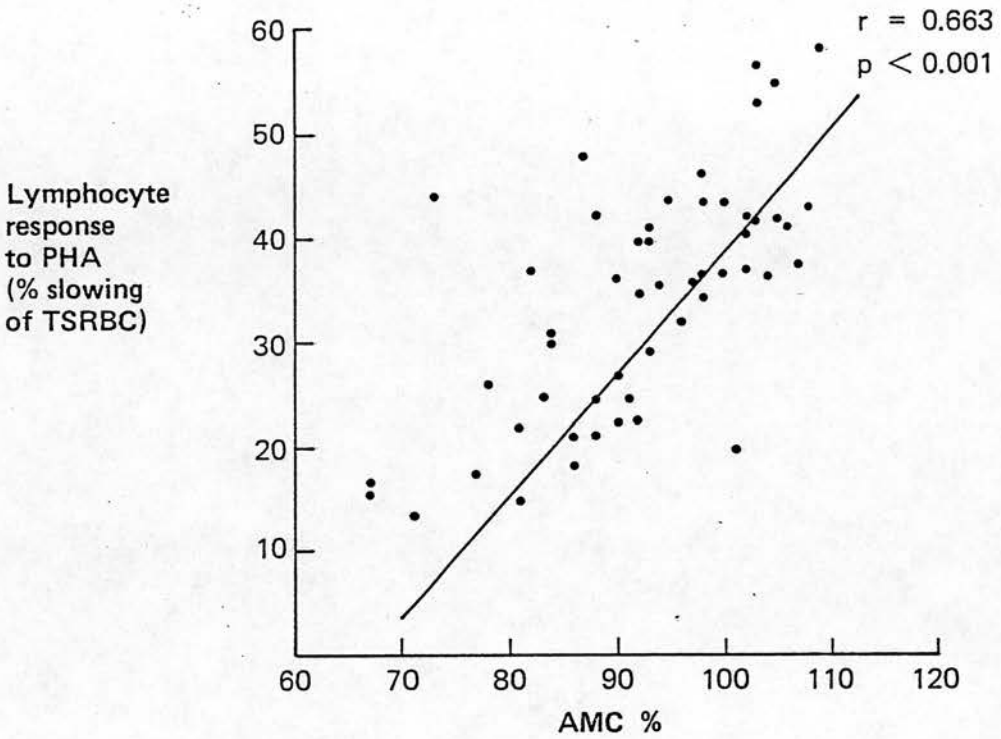


Figure 3.9 Correlation of lymphocyte responses to PHA with arm muscle circumference (AMC) as a percentage of normal in patients with benign disease

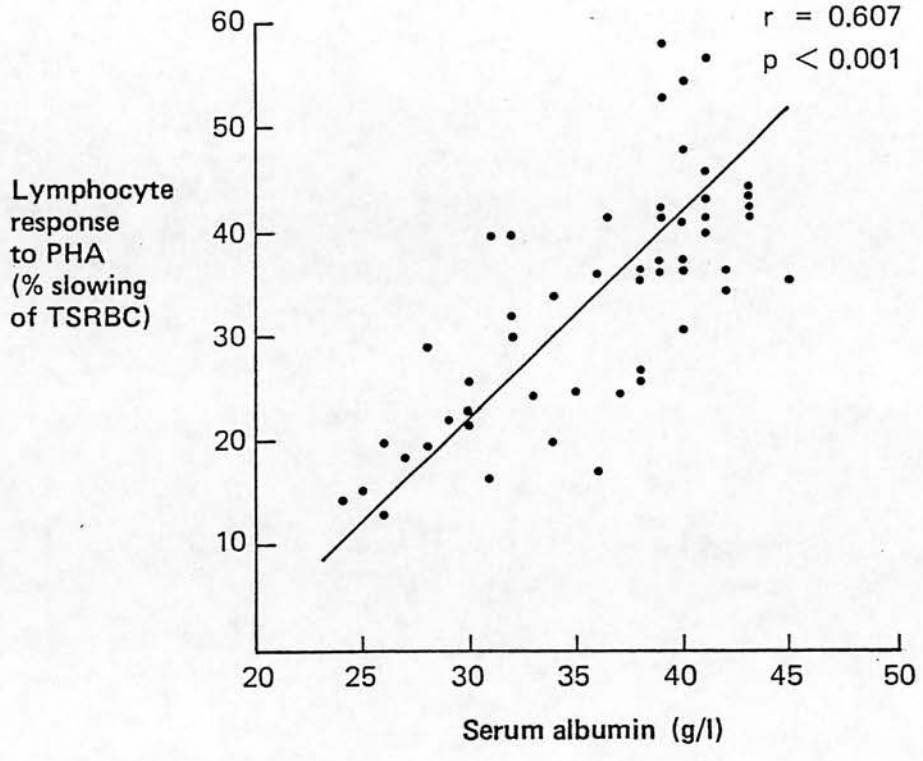


Figure 3.10 Correlation of lymphocyte responses to PHA with serum albumin concentrations in patients with benign disease

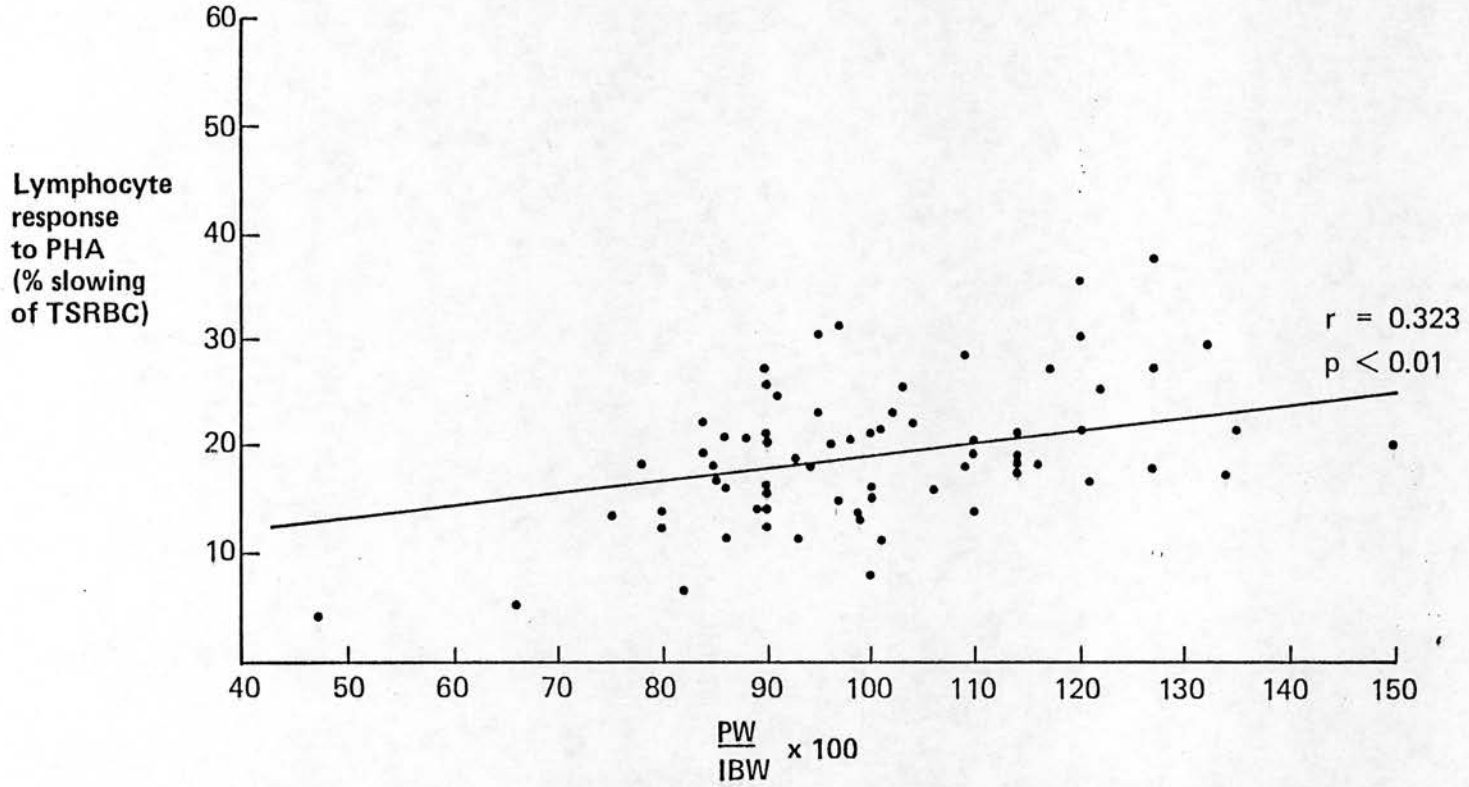


Figure 3.11 Correlation of lymphocyte responses to PHA with percentage weight for height in patients with malignant disease

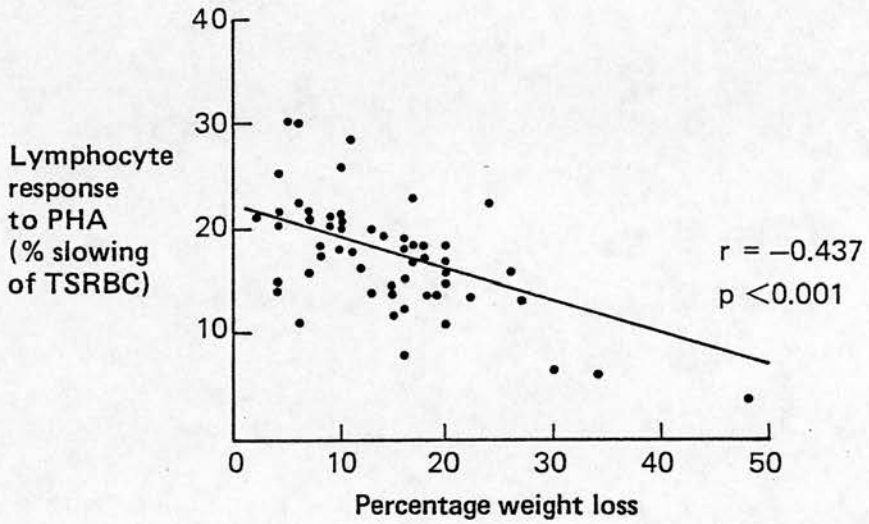


Figure 3.12 Correlation of lymphocyte responses to PHA with percentage weight loss in patients with malignant disease

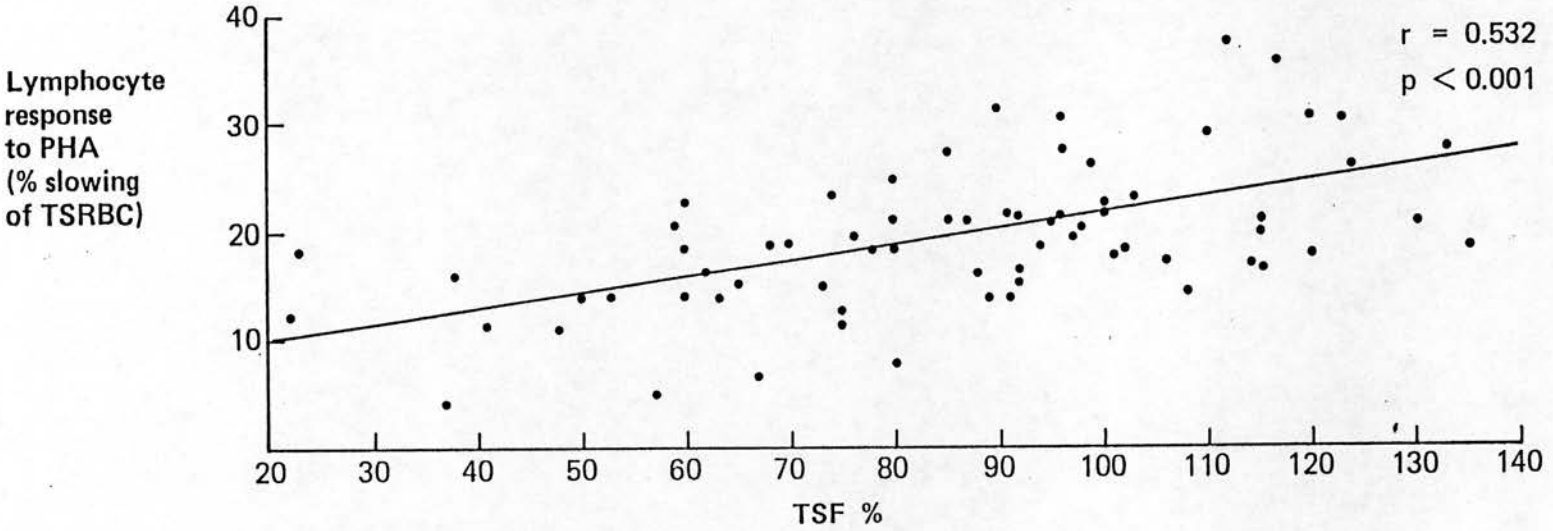


Figure 3.13 Correlation of lymphocyte responses to PHA with triceps skin fold (TSF) thickness as a percentage of normal in patients with malignant disease

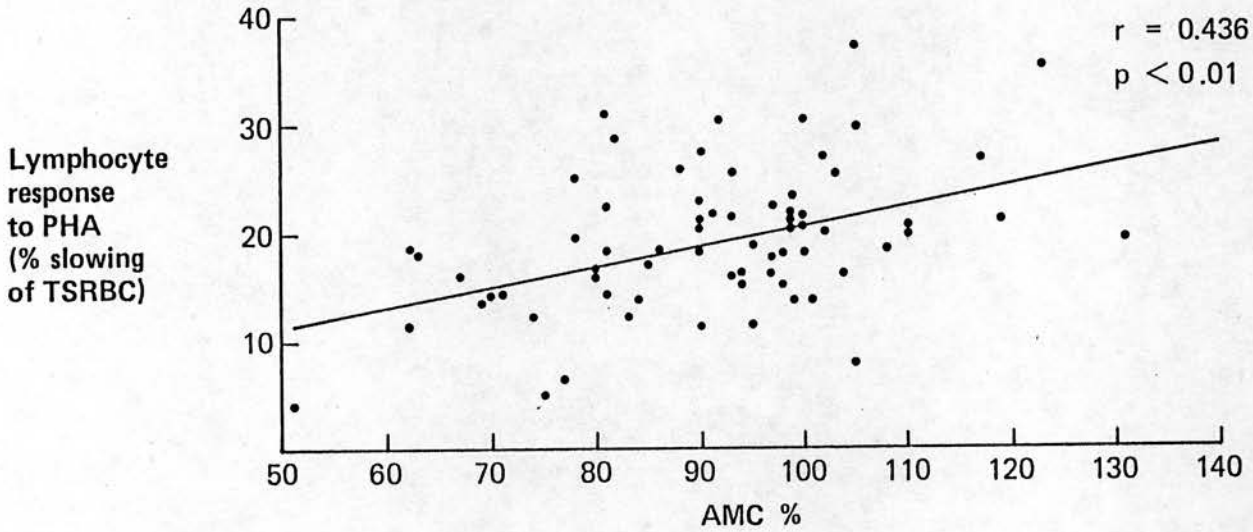


Figure 3.14 Correlation of lymphocyte responses to PHA with arm muscle circumference (AMC) as a percentage of normal in patients with malignant disease

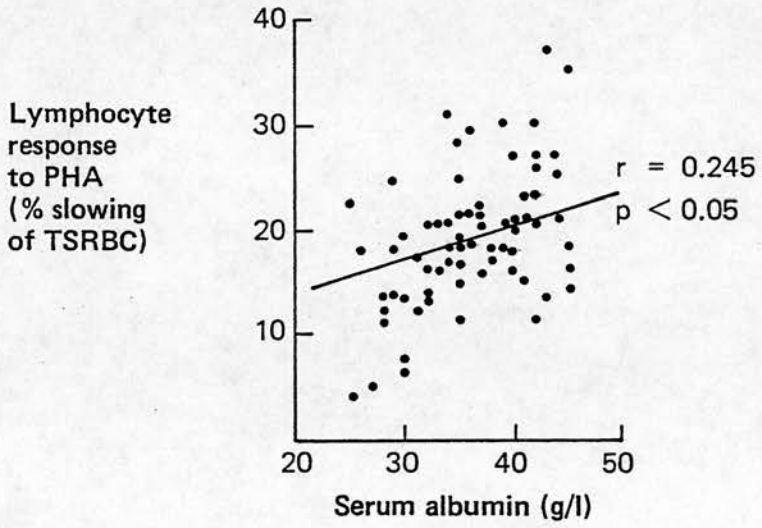


Figure 3.15 Correlation of lymphocyte responses to PHA with serum albumin concentrations in patients with malignant disease

correlations, although significant, were weak.

Correlations of lymphocyte responses to PPD and PHA with nutritional parameters in patients with malignancy (Table 3.7) were statistically significant but of poor sensitivity, but were frequently not significant for Con A and PWM. The strongest correlation was between the response to PPD and weight loss ($r = 0.55$, $p<0.001$).

3.2.2 (c) Discussion

Correlation between in vitro lymphocyte reactivity and nutritional parameters could not be identified for normal subjects because of the relatively narrow range of results of lymphocyte reactivity and the absence of nutritional impairment. Generally, all subjects had high lymphocyte reactivity and all had normal nutritional assessments. It was observed that some individual nutritional parameters differed from standard values in a few subjects because of idiosyncratic variation in the size of the measured body compartments (TSF, AMC, serum albumin) or in overall body weight (PW/IBW).

Nutritional deficiency was associated frequently with poor lymphocyte reactivity in patients with benign diseases; whereas in the absence of objective evidence of malnutrition, lymphocyte reactivity was similar to that observed in healthy subjects.

The nutritional parameters of weight for height (PW/IBW) had the weakest correlations with lymphocyte reactivity in benign patients. This finding may be due partly to the use of standards of weight for height which may not have reflected accurately the average values of the local population. Also, weight for height measurements give no indication of changes in nutritional status and do not take account of the previously obese patient who has lost a significant amount of body weight.

The results show that in malignant disease lymphocyte responses to PPD antigen and particularly mitogens correlated poorly with nutritional status. Severe nutritional impairment adversely affected lymphocyte reactivity in patients with malignant disease, a situation also seen with benign disease. The nearer the nutritional parameters were to standard values, the wider was the scatter of responses, suggesting that factors other than nutrition influenced lymphocyte reactivity in some patients with cancer.

Percentage weight loss $\frac{(UW-PW\ \%)}{UW}$ showed the best correlation with lymphocyte reactivity in the malignant group and was the only parameter to exhibit significant correlations with all four lymphocyte stimulants. The weight loss index measures illness-related weight loss and does not differentiate between particular body compartments which may be depleted to different degrees. Not surprisingly, the sensitivity of this index was poor but the results indicate that recent or acute malnutrition was associated with poor lymphocyte reactivity in these cancer patients. In benign patients the same index of percentage weight loss correlated well with lymphocyte reactivity but the association of impaired lymphocyte responsiveness with poor nutritional status was stronger in the benign group anyway.

It is of interest to note that illness-related weight loss was detected in 25 of 52 benign patients (48%) and 55 of 69 cancer patients (79%). Although neither group was a consecutive series, patients were not selected on the basis of nutrition status, and the finding of a high incidence of weight loss among patients with benign as well as malignant disease draws attention to the frequency of nutritional problems in patients in a general surgical ward. It also draws attention to the need

to define and control for the nutritional status of patients involved in immunological research.

3.3 DEFINITION OF MALNUTRITION

It was necessary to define "malnutrition" for the purpose of this study. The problems of defining malnutrition have been reviewed in Section 1.6.2 and, realising the inherent faults in the methods of nutritional assessment, it was decided to use the five nutritional parameters described in Section 2.2, (weight-for- height, weight loss, arm muscle circumference, triceps skin fold thickness and serum albumin). The "85% cut-off level" was used - that is, an anthropometric measurement was regarded as indicating significant nutritional impairment if it was less than 85% of the standard value; weight loss of greater than 15% of usual or pre-illness weight was taken to indicate significant nutritional impairment. A serum albumin concentration below the lower limit of the laboratory reference range (30 - 50 g/l) was regarded as an index of significant nutritional deficit.

The distribution of abnormally low nutritional parameters is shown in Table 3.8. 62% of patients had less than two abnormal parameters, 5% had two and 33% had more than two. It was decided to classify patients as malnourished if the majority (three or more) of their parameters were abnormally low. Using this criterion none of the healthy group, 18 (35%) of the benign group and 22 (32%) of the malignant group were classified as malnourished.

3.3.1 LYMPHOCYTE RESPONSES IN WELL NOURISHED AND MALNOURISHED SUBJECTS

Lymphocyte responses to PPD, PHA, Con A, and PWM were analysed for

Table 3.8 Distribution of abnormal anthropometric and biochemical parameters in patients with benign and malignant diseases

NUMBER OF ABNORMALLY LOW NUTRITIONAL PARAMETERS	NUMBER OF PATIENTS		PERCENTAGE OF TOTAL
	Benign	Malignant	
0	23	26	40%
1	7	19	22%
2	4	2	5%
3	10	9	16%
4	4	6	8%
5	4	7	9%
Total	52	69	100%

the five groups of subjects: (1) normal, (2) benign well nourished, (3) benign malnourished, (4) malignant well nourished, and (5) malignant malnourished.

Scatter diagrams were constructed for lymphocyte responses. An example is given in Figure 3.16. There was a wide range of responses in each subject group. All groups overlapped except the normal group did not overlap with the malignant malnourished group. There was no significant difference between responses to PHA of lymphocytes from normal subjects and well nourished patients with benign disease. Comparison of benign well nourished and benign malnourished groups showed a highly significant difference between responses ($p<0.001$). The malignant malnourished group had significantly lower lymphocyte responses to PHA than the malignant well nourished group ($p<0.001$). Responses of the two benign groups were significantly higher than the responses of their malignant counterparts ($p<0.001$ in each case). Lymphocyte responses to PPD, Con A and PWM produced similar patterns of distribution as responses to PHA. For each stimulant, comparison of the two benign and malignant groups revealed statistically significant differences between the lymphocyte responses of well nourished and malnourished patients ($p<0.001$). Similarly, there was no significant difference between normal subjects and benign well nourished patients.

These experiments show that malnutrition secondary to a disease process suppresses lymphocyte reactivity. Even in conditions known to adversely affect immune function, such as cancer, the influence of malnutrition on lymphocyte reactivity was demonstrated clearly. Lymphocyte reactivity could be correlated with nutritional impairment as identified by these anthropometric and biochemical methods of assessment in patients with benign and malignant disorders. Generally, the association of reduced

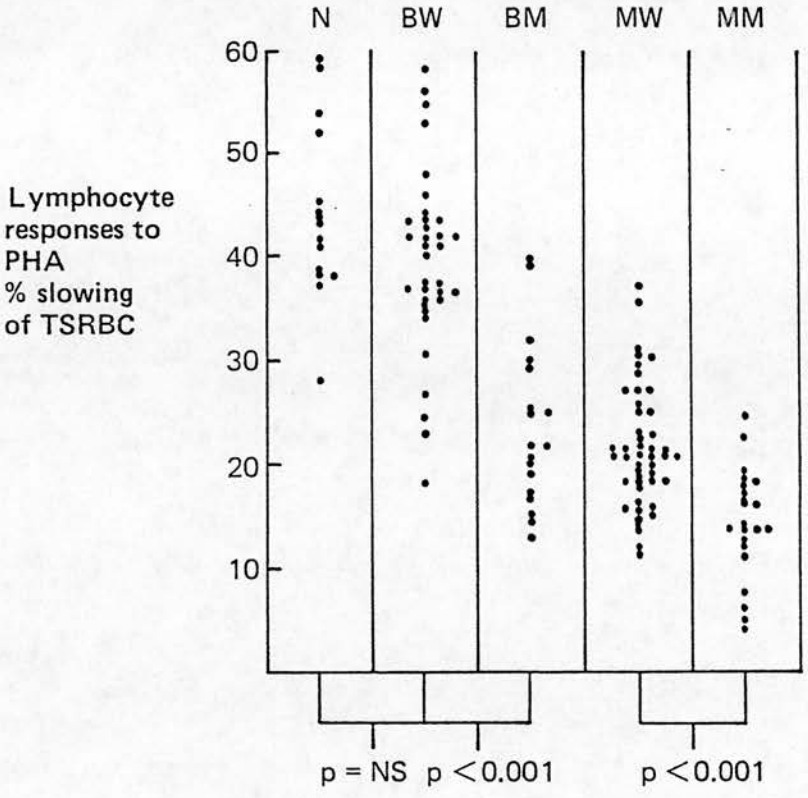


Figure 3.16 The results of <u>in vitro</u> lymphocyte responses to PHA of each of the five groups of subjects classified by nutritional status

N = Normal
BW = Benign well nourished
BM = Benign malnourished
MW = Malignant well nourished
MM = Malignant malnourished

lymphocyte activity and malnutrition was stronger in the benign group of patients, suggesting that additional factors influenced lymphocyte responses in patients with malignancy. Definition of nutritional impairment by five simple criteria allowed two sub-groups of benign and malignant patients to be identified.

Lymphocyte responses of the benign well nourished group did not differ significantly from those of the healthy group although the responses of some benign patients were below the range of results of the normals (Figure 3.16). These low results could have been due to the influence of the disease process or the failure of the nutritional assessment to detect significant nutritional deficit which adversely affected lymphocyte reactivity.

Problems relating to the definition of the malnourished state have been discussed in Section 1.6.2. The present definition used (a) both anthropometric and biochemical methods of assessment, (b) an 85% cut off level to define a result as being abnormally low, and (c) a 'majority rule' to classify patients as malnourished. Clearly, this definition is still open to criticism about the methods of assessment, but it proved to be a clinically useful definition, and its validity was supported by the results reported in this section.

The study highlights the influence of nutrition on <u>in vitro</u> reactivity of lymphocytes in patients encountered in hospital practice. Such patients (especially those with malignancy) are frequently included in immunological research, and the present results indicate that close attention must be paid to the nutritional status of patients when interpreting tests of immune function.

3.4 PLASMA LYMPHOCYTOTOXICITY

All human plasma samples were examined for the presence of lymphocytotoxic antibodies. Lymphocytotoxicity tests were performed as described in Section 2.4. No plasma sample had cytotoxic activity against the panel of lymphocytes, thereby excluding the presence of pre-formed lymphocytotoxins as a cause of impaired lymphocyte reactivity.

3.5 STUDIES OF PLASMA SUPPRESSIVE ACTIVITY

Plasma suppressive activity (PSA) refers to the ability of a plasma sample to suppress or inhibit the reactivity of lymphocytes following antigenic or mitogenic stimulation.

A study was undertaken to examine the SA of plasma from cancer patients in an attempt to explain the immunological impairment so frequently observed in cancer patients (Section 1.4). PSA was assessed using autologous and allogeneic lymphocytes.

3.5.1 AUTOLOGOUS PSA

3.5.1 (a) Method

PSA was measured against each patient's own lymphocytes using the TEEM test method (Section 2.3.4). Lymphocytes and plasma samples were prepared as described in Section 2.3. Patients were classified into five groups as described before (normal, benign well nourished, benign malnourished, malignant well nourished, or malignant malnourished) according to the previously adopted criteria (Sections 2.1 and 3.3). The Mann Whitney U test was used for statistical analysis.

3.5.1 (b) Results

Results of PSA against autologous lymphocytes are shown in

Figure 3.17 and Table 3.9. PSA of the normal group had a relatively small range, (27.6 - 36.7 μl plasma), and the median was 32.0 μl plasma.

There was a wide range of results in the benign well nourished group, with a skew towards the lower values. The group did not differ significantly from the normal group. There was some overlap between the two benign groups but generally the PSA of malnourished patients with benign disease was high, as indicated by the relatively small microlitre volumes of plasma. PSA's of the two benign groups were significantly different ($p<0.001$).

Plasma suppressive activity was high in well nourished and malnourished patients with cancer and there was no statistically significant difference between the two groups. The PSA of each malignant group was significantly greater than the benign and normal groups, the median PSA's being 2.01 μl plasma (well nourished) and 1.96 μl plasma (malnourished).

3.5.1 (c) Discussion

The results demonstrate that all plasma samples, even those from healthy subjects, contained some SA, suggesting that plasma factors have a role in health and disease in the regulation of lymphocyte activity. It appears to be a natural phenomenon as it was observed using untreated autologous lymphoctyes in all subjects. This important observation points to a possible mechanism of immune regulation and will be examined in greater detail later.

Malnourished patients with benign disease had a significant increase in PSA which could have been due to their impaired nutritional status or their disease. The well nourished and malnourished benign

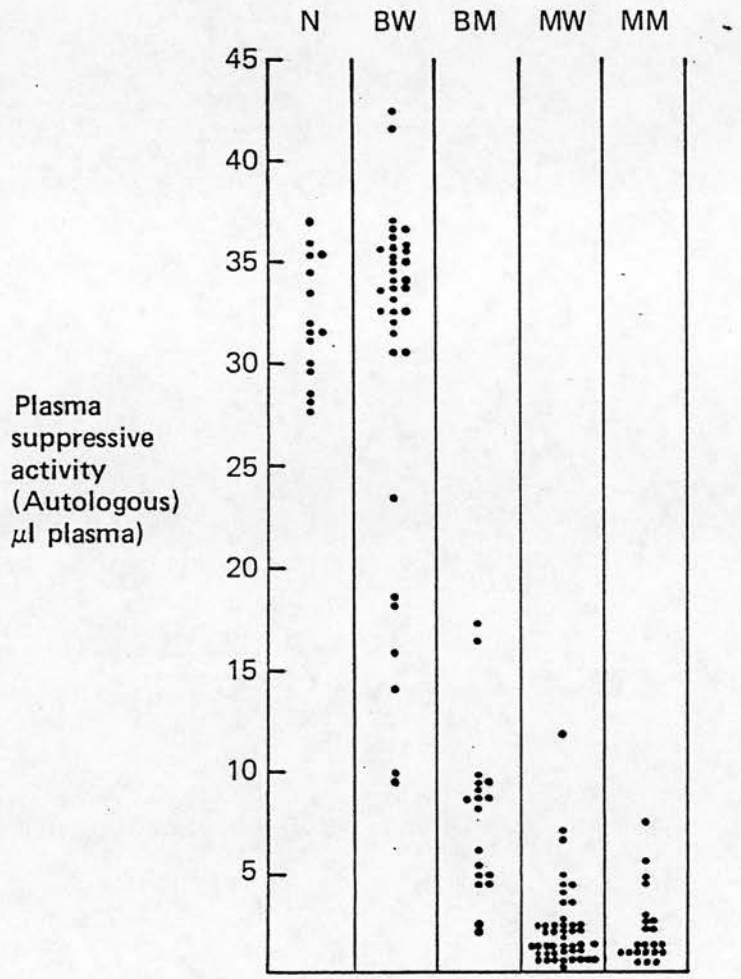


Figure 3.17 Plasma suppressive activities (PSA) against autologous lymphocytes

N = Normal
BW = Benign well nourished
BM = Benign malnourished
MW = Malignant well nourished
MM = Malignant malnourished

Table 3.9 Plasma suppressive activities against autologous lymphocytes according to nutritional status

GROUP	NUTRITIONAL STATUS	n	PSA (median)	p VALUE*
Normal	Well nourished	15	32.0	NS
Benign	Well nourished	34	33.8	< 0.001
Benign	Malnourished	18	7.7	
Malignant	Well nourished	47	2.01	NS
Malignant	Malnourished	22	1.96	

PSA = Plasma suppressive activity (μl plasma)

NS = Not significant

* = Mann Whitney U test

patients could not be matched in terms of their disease because their nutritional status was secondary to their disease, and so it cannot be concluded that their increased PSA was due solely to impaired nutrition.

The importance of the disease process cannot be ignored. It can be seen in Figure 3.17 that the lowest seven PSA values of the benign well nourished group were all below the lowest PSA value of the normal group; closer examination of these seven patients showed that six had a degree of illness related weight loss, although in only three did it amount to 10% or more of body weight. Five of the seven had inflammatory bowel disease and this may well be relevant to the finding of increased plasma suppressive activity. The other two patients had fibroadenosis of the breast and a villous adenoma of the rectum. The possible importance of an increased PSA in these patients is discussed in Chapter 6.

The differences in PSA's of the two benign groups is striking, and it is unlikely that they can be explained on the basis of disease alone. Two patients in the benign malnourished group had long-standing oesophageal strictures with little inflammatory reaction and so presumably the complicating factor of their underlying disease would be minimal; both patients had high PSA values.

The SA of any plasma sample depends on its inherent suppressive ability as well as the strength of the lymphocyte response which the plasma has to suppress. The use of autologous lymphocytes was considered to be a more accurate reflection of the <u>in vivo</u> situation than the use of allogeneic lymphocytes.

3.5.2 ALLOGENEIC PSA

Because measurement of PSA relies on the ability of plasma to suppress a lymphocyte response, it is important to standardise the

lymphocyte response for studies concerned with identifying and measuring suppressive plasma factors. If lymphocyte responses were not standardised then a sample of plasma would have different PSA values according to the strength of the responses that it had to suppress.

3.5.2 (a) Methods

Lymphocytes were obtained from a panel of three healthy adult male donors who had kindly donated blood samples to the laboratory on numerous occasions during the three year period. The reactivity to PPD of lymphocytes from these donors was remarkably similar and consistent during this time (Appendix 13). Lymphocytes were prepared as described earlier but cells from different donors were never mixed. PSA was estimated as described previously.

3.5.2 (b) Results

The pattern of PSA against allogeneic lymphocytes was broadly similar to that of PSA against autologous lymphocytes (Figure 3.18 and Table 3.10). The normal group was not significantly different from the benign well nourished group, which had significantly lower PSA's than the benign malnourished group ($p<0.001$). PSA was increased in six well nourished benign patients who also had raised PSA against autologous lymphocytes. Two malnourished patients had values of PSA against allogeneic lymphocytes which fell within the range of PSA's of the normal group. These two patients had high PSA's when tested against autologous lymphocytes, suggesting that the apparently high 'autologous PSA' was due to poor lymphocyte reactivity rather than a true effect of their plasma. PSA was high in patients with malignant disease and there was no

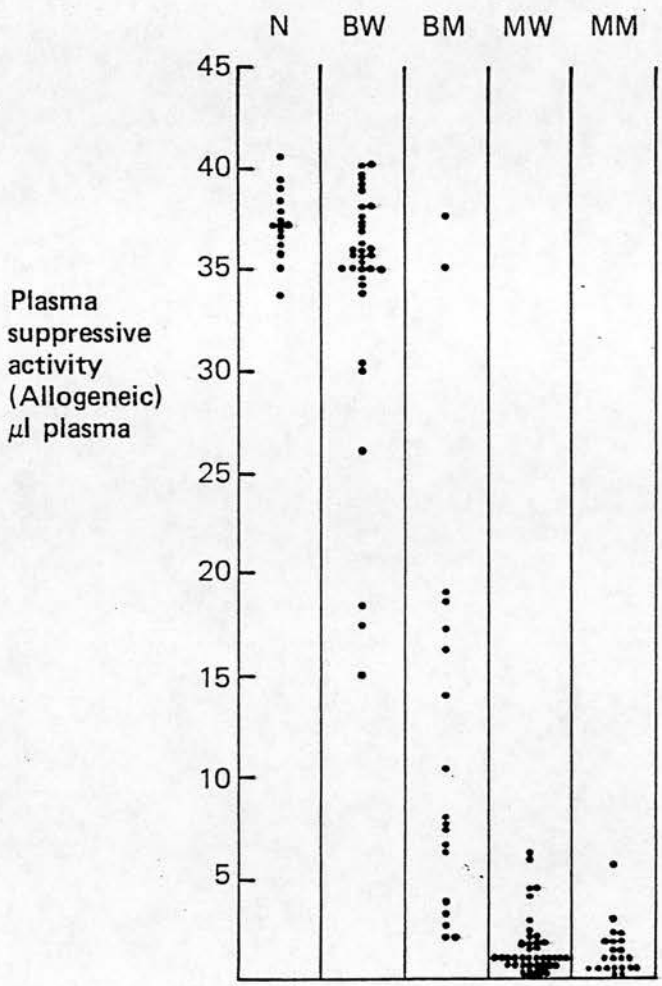


Figure 3.18 Plasma suppressive activities (PSA) against allogeneic lymphocytes

N = Normal
BW = Benign well nourished
BM = Benign malnourished
MW = Malignant well nourished
MM = Malignant malnourished

Table 3.10 Plasma suppressive activities against allogeneic lymphocytes according to nutritional status

GROUP	NUTRITIONAL STATUS	n	PSA (median)	p VALUE*
Normal	Well nourished	15	37.1	NS
Benign	Well nourished	34	35.3	< 0.001
Benign	Malnourished	18	7.6	
Malignant	Well nourished	47	1.63	NS
Malignant	Malnourished	22	1.29	

PSA = Plasma suppressive activity (μl plasma)

NS = Not significant

* = Student's t test

significant difference between the PSA's of well nourished and malnourished patients with malignant disease.

3.5.2 (c) Discussion

The use of allogeneic lymphocytes indicates that SA in plasma is mainly non-specific as it is able to inhibit both autologous and allogeneic cells. Also, it is clear that the high PSA of patients who are malnourished or who have malignant disease is actually due to factors within plasma and not simply due to reduced lymphocyte reactivity alone.

It is of interest to compare the PSA results with the two different lymphocyte populations for the five subject groups (Tables 3.9 and 3.10). Allogeneic PSA was significantly lower (higher μl volume) than autologous PSA in normal subjects and in benign well nourished subjects, the latter group behaving very similarly to normal subjects with respect to lymphocyte reactivity (Section 3.3.1) and PSA. There was also a reduction in mean PSA in the benign malnourished with the use of allogeneic cells; the difference did not reach statistical significance. PSA values of this group were scattered widely. Allogeneic PSA was greater (smaller μl volume of plasma) than autologous PSA in both groups of malignant patients. This last observation is intriguing, as it implies that there are plasma factors in cancer patients which are suppressive for allogeneic but not autologous lymphocytes. The differences are even greater than the PSA values lead one to believe, as the allogeneic lymphocyte responses are significantly stronger than the responses of lymphocytes from cancer patients (Section 3.3).

3.5.3 INFLUENCE OF NUTRITIONAL STATUS IN PATIENTS WITH BENIGN DISEASE

The PSA studies indicate that PSA against autologous and allogeneic

cells is related to nutritional status in patients with benign disease but not in cancer patients.

The relationship between PSA and nutrition was quantified by calculating a correlation coefficient (r) and value of p for each set of data from the benign group of patients (Table 3.11). Correlations of nutritional parameters with autologous and allogeneic PSA were generally strong and all were significant ($p<0.001$). Correlations were stronger with PSA against allogeneic rather than autologous cells for four of the five nutritional parameters. Correlations of percentage weight loss with autologous and allogeneic PSA values are shown in Figures 3.19 and 3.20 respectively as examples. It is of interest to note that correlations of PSA with serum albumin were not only statistically significant ($p<0.001$) but were the strongest ($r = 0.83$ (autologous) and $r = 0.78$ (allogeneic)).

The fact that all nutritional parameters correlated with PSA suggests strongly that is was nutritional impairment rather than "disease factors" that increased SA in these patients with benign disease. However, these observations do not prove this contention; to do so, it would have been necessary to measure PSA in a series of patients with the same disorder of exactly the same extent, duration and severity but with varying degrees of nutritional impairment. Such a situation is rarely found in clinical practice and would have required a considerable time in order to collect a sufficiently large number of similar patients.

3.5.4 INFLUENCE OF THE STAGE OF MALIGNANT DISEASE

Malignant disease is associated with an increase in the SA of plasma (Sections 3.5.1 and 3.5.2). The PSA results of the 69 patients with malignancy were analysed on the basis of the stage of disease to determine

Table 3.11 Correlations of plasma suppressive activity with nutritional assessment of patients with benign disease

NUTRITIONAL PARAMETER	n	COEFFICIENT OF CORRELATION (r)*	
		Autologous PSA	Allogeneic PSA
Weight for height	55	0.71	0.77
Weight loss	25	-0.69	-0.76
Triceps skin fold	55	0.72	0.84
Arm muscle circumference	55	0.53	0.59
Serum albumin	55	0.83	0.78

* All correlations were statistically significant at $p < 0.001$

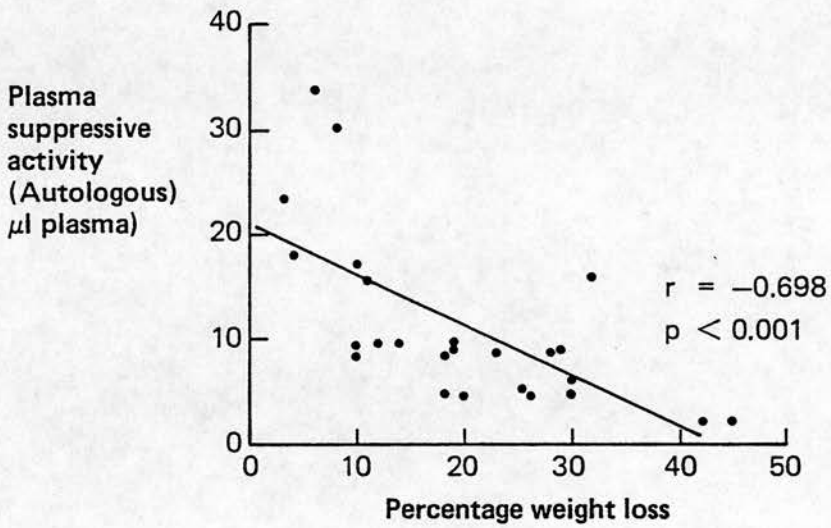


Figure 3.19 Correlation of plasma suppressive activity (PSA) against autologous lymphocytes with percentage weight loss in patients with benign disease

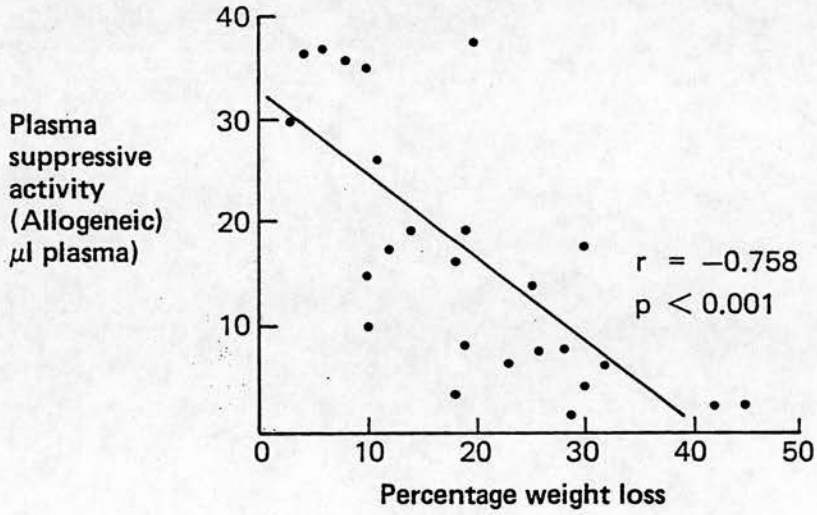


Figure 3.20 Correlation of plasma suppressive activity (PSA) against allogeneic lymphocytes with percentage weight loss in patients with benign disease

whether PSA was related to the amount of malignant tissue. Malignant diseases were staged according to clinical staging criteria (Appendix 1).

There were four patients with Stage A disease, 21 Stage B, 19 Stage C and 25 Stage D. The PSA results have been shown in Figure 3.21 as logs of microlitre volumes of plasma required to cause 50% inhibition of the responses of autologous lymphocytes. There was no statistically significant difference between the four groups. Comparison of PSA values of Stage A and B patients just failed to reach statistical significance ($p=0.06$, Mann Whitney U test). The number of patients with Stage A disease was small and so the data could be subject to a type II, or beta, statistical error in which the small number of subjects has obscured a true difference. While there is, perhaps, a trend towards a lower PSA in early disease, there was no correlation between PSA values and Stages B, C and D. To establish the presence of an association between early disease and low PSA it would be necessary to study, firstly, a larger number of patients and, secondly, to study patients with early stages of a variety of different malignancies to exclude the influence of disease specificity. All of the Stage A patients in this study had carcinoma of the breast.

The failure to demonstrate a correlation between PSA and stage of disease could be due to the nature of the suppressive substances, or their effect on lymphocytes within the TEEM test system, or the relative crudity of the staging system. If SA is due to substances released from the surface of tumour cells, then it would be expected that PSA would be proportional to tumour bulk. If, however, SA was the result of interaction of host factors with tumour-related factors, then SA would depend on the amount of or functional capacity of the host factors in the presence of an abundance of tumour-related factors. Thus, the present result is consistent with the initial hypothesis (Section 1.8).

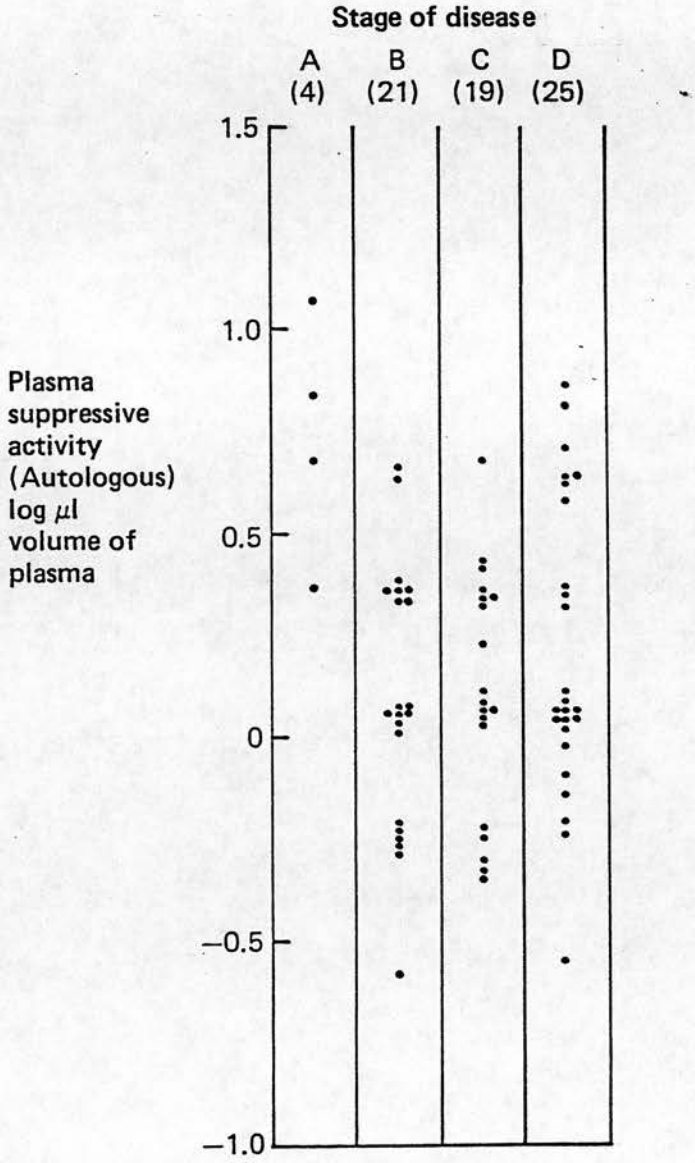


Figure 3.21 Plasma suppressive activities and the stage of disease in 69 patients with malignant disease

The inability to estimate adequately the volume of malignant disease is a long-standing problem in oncology. Most staging systems use criteria which can be related to prognosis, which may not necessarily depend on the absolute volume of malignant tissue but rather the strategic location of malignant deposits. Clearly, the extent of a malignant disease may be very varied within each of the four clinical stages, and inadequacy of currently available staging systems could contribute to the apparent failure to correlate PSA with the extent of disease. Alternatively, the existence of PSA in malignant disease could be an "all or none" phenomenon, in which case all malignancies irrespective of stage would be associated with a high PSA. Further studies are required to investigate the factors responsible for SA before this explanation can be accepted.

3.5.5 INFLUENCE OF THE SITE OF MALIGNANT DISEASE

An attempt was made to identify an association between PSA and the primary site of malignant disease. Scatter diagrams were drawn by plotting PSA values for each patient with a particular malignancy. No consistent pattern was found for either autologous or allogeneic PSA, except that the group of patients with carcinomas of the breast had a slightly lower mean PSA than the other groups. However, the breast group contained four patients with Stage A disease whereas no other malignant group had any patients with Stage A tumours. After excluding patients with carcinoma of the breast, the PSA's of patients with other primary sites of cancer were not significantly different.

3.5.6 SUMMARY

The plasma of all subjects had the ability to suppress to some degree <u>in vitro</u> responses of autologous and allogeneic lymphocytes to

antigenic stimulation. This finding is consistent with the view that there is a factor, or factors, in plasma which regulates the activity of lymphocytes in relation to their responses to stimuli. Suppressive activity was increased in patients with benign diseases, such as inflammatory bowel disease, and in patients who were malnourished as a result of a benign disease. PSA was increased significantly in patients with malignant disease, where the influence of malnutrition on PSA was not apparent. There was a suggestion that PSA might not be quite so high in patients with early malignancies as in patients with advanced disease, although the number of patients with early cancer in this study was too small for a definite conclusion to be made.

3.6 IDENTIFICATION OF PLASMA SUPPRESSIVE FACTORS

Studies were performed to identify the suppressive factor or factors in plasma. Experiments were designed to identify the suppressive factor(s) in normal subjects and to determine whether or not the same factor(s) was supppressive in patients in whom PSA was raised, and whether or not this factor(s) was quantitatively or qualitatively different in such patients. A search was also made for additional suppressive factors in patients with raised PSA.

3.6.1 COLUMN FRACTIONATION OF PLASMA

3.6.1 (a) Method

Plasma samples were obtained from five groups of subjects: 1. healthy adult volunteers (n=5); 2. well nourished patients with benign disease (n=5); 3. malnourished patients with benign disease (n=5); 4. well nourished patients with malignant disease (n=6); and 5. malnourished

patients with malignant disease (n=6). Details of the 22 patients are given in Table 3.12. An attempt was made to study patients with disorders of the same viscera; e.g. four patients with breast disorders, two with fibroadenosis and two with carcinoma. The patient groups were well matched for age and sex distribution, but were all significantly older than the group of healthy volunteers. Plasma samples (2.0 ml) were fractionated by gel filtration (Section 2.5). Each fraction of plasma was analysed for (1) total protein content by spectrophotometry, (2) SA against autologous and allogeneic lymphocytes, using the TEEM test (Section 2.3), and (3) constituent proteins as determined by fused rocket immunoelectrophoresis (Section 2.6).

Thus, four sets of results were obtained from each plasma sample as a result of gel filtration - protein content, SA (autologous), SA (allogeneic) and a pattern of protein distribution. These results were expressed graphically for each plasma sample and one representative example from each of the five subject groups is shown in Figures 3.22 - 3.26. The void volume of the gel filtration column was collected in the first 20 - 22 fractions, and these were discarded and not represented in Figures 3.22 - 3.26. The first fractions to be eluted from the column are represented on the left of the Figures and the later fractions are towards the right.

At the uppermost part of each Figure is the plot of optical density at 280 nm. This continuous line represents the tracing produced by the chart recorder of the spectrophotometer. The two histograms represent fractions of plasma in which SA against either autologous or allogeneic lymphocytes was detected. SA was quantified by adding 0.5 ml of each plasma fraction to 2.5 ml Hank's BSS (Appendix 4) which was then incubated with 0.5×10^6 autologous or allogeneic lymphocytes for 30 minutes, after which the TEEM test method was followed (Section 2.3.3). Each vertical bar

Table 3.12 Details of the 22 patients studied in Section 3.6

GROUP	NUMBER	AGE (mean ± 1SD)	SEX (M:F)	DIAGNOSIS
Normal	5	38.6 ± 9.1	3:2	-
Benign well nourished	5	58.2 ± 10.8	3:2	Fibro-adenosis of breast (2) Diverticular disease (2) Peptic ulcer (1)
Benign malnourished	5	59.4 ± 8.8	3:2	Chronic pancreatitis (1) Ulcerative colitis (2) Oesophageal stricture (2)
Malignant well nourished	6	63.5 ± 4.3	3:3	Breast (2) Colon (3) Stomach (1)
Malignant malnourished	6	64.2 ± 7.4	3:3	Pancreas (2) Colon (2) Stomach (2)

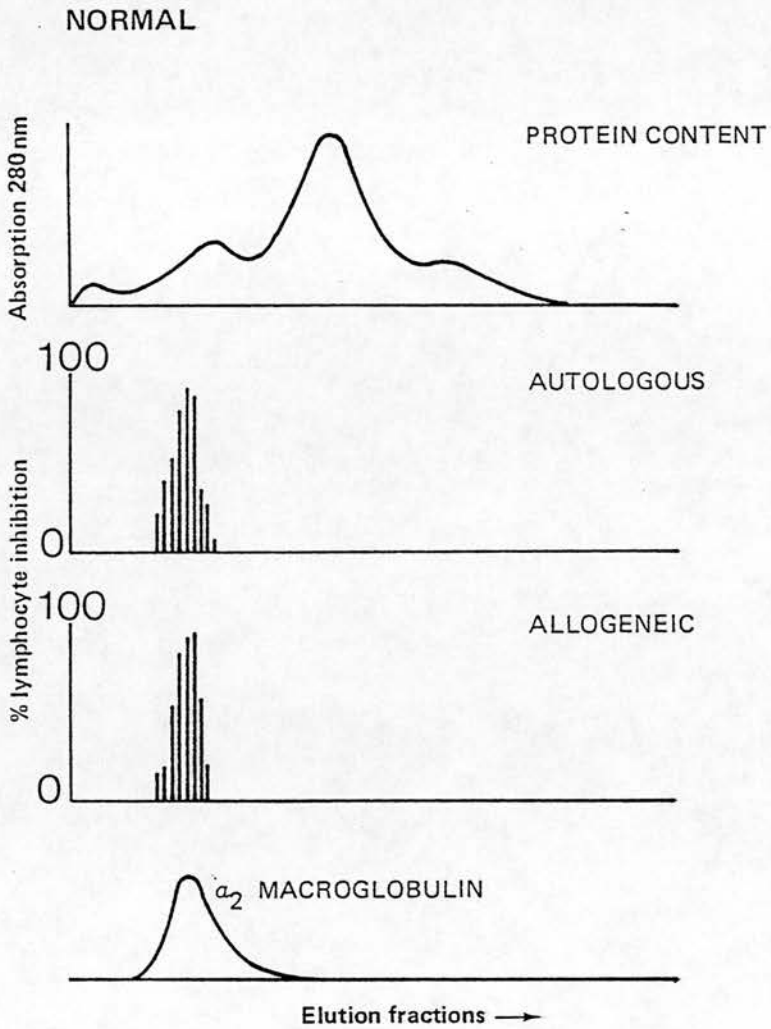


Figure 3.22 An example of the optical density, percentage inhibition of autologous and allogeneic lymphocytes and distribution of alpha 2 macroglobulin in the plasma fractions of a normal subject

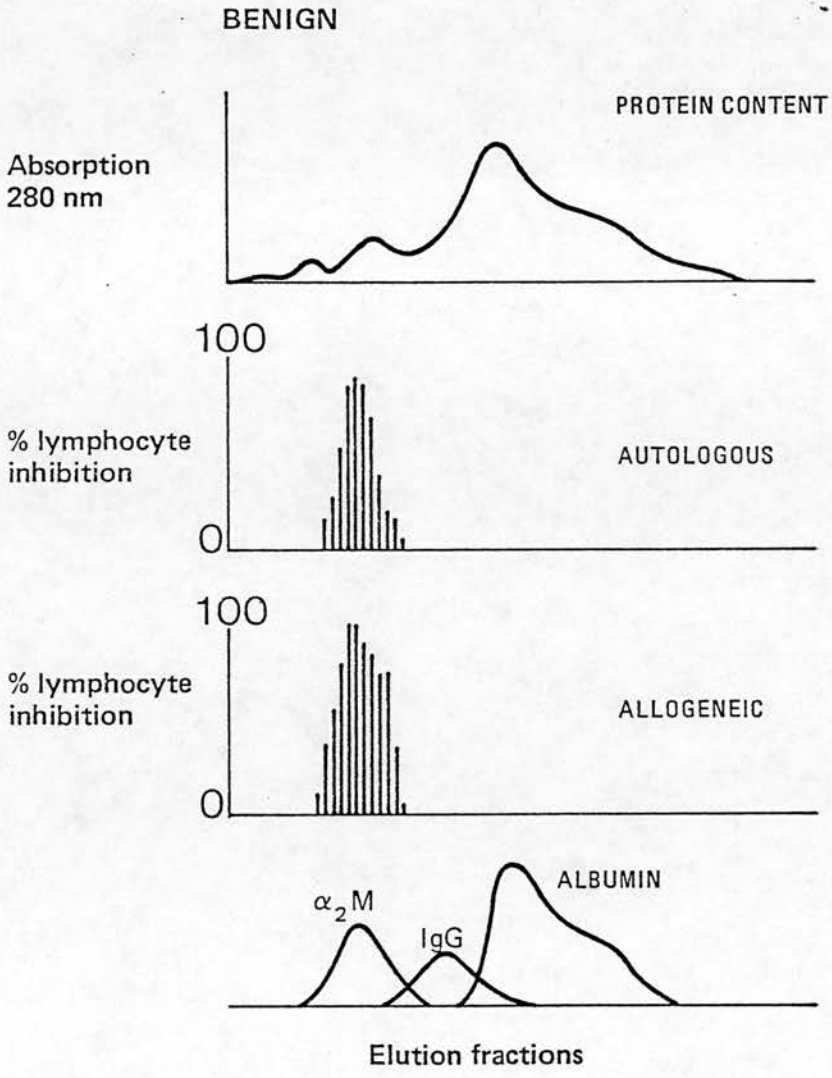


Figure 3.23 An example of the optical density, percentage inhibition of autologous and allogeneic lymphocytes and distribution of alpha 2 macroglobulin, IgG and albumin in the plasma fractions of a patient with a benign gastric ulcer

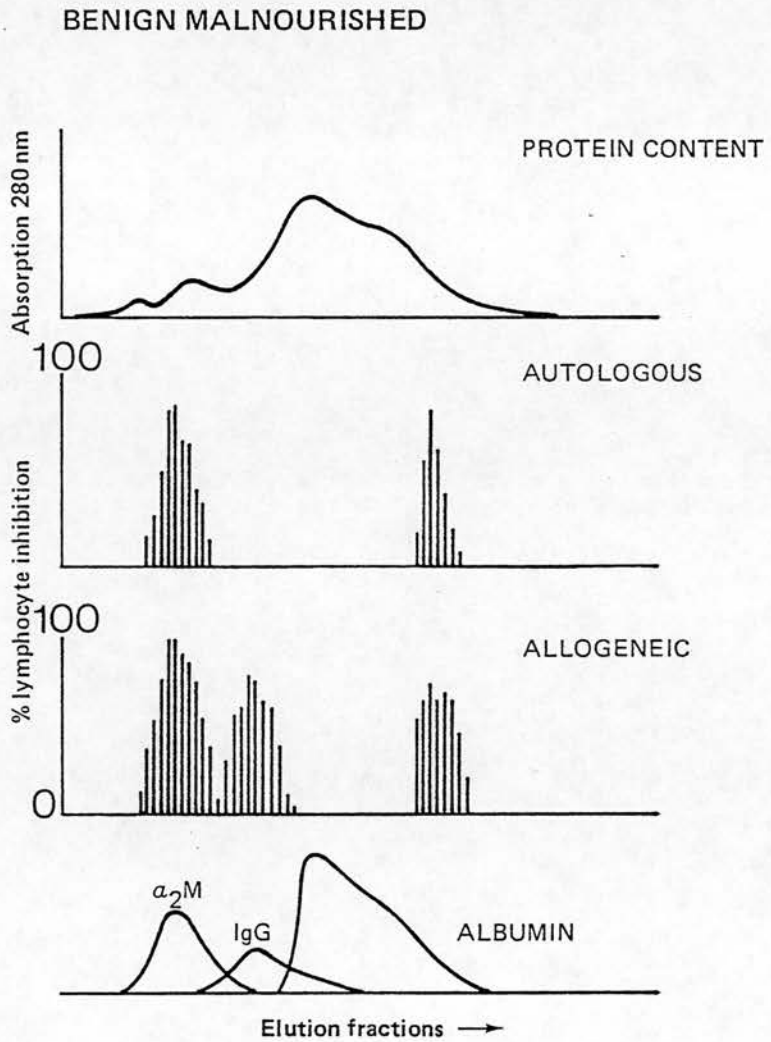


Figure 3.24 An example of the optical density, percentage inhibition of autologous and allogeneic lymphocytes and distribution of alpha 2 macroglobulin, IgG and albumin in the plasma fractions a malnourished patient with a benign oesophageal stricture

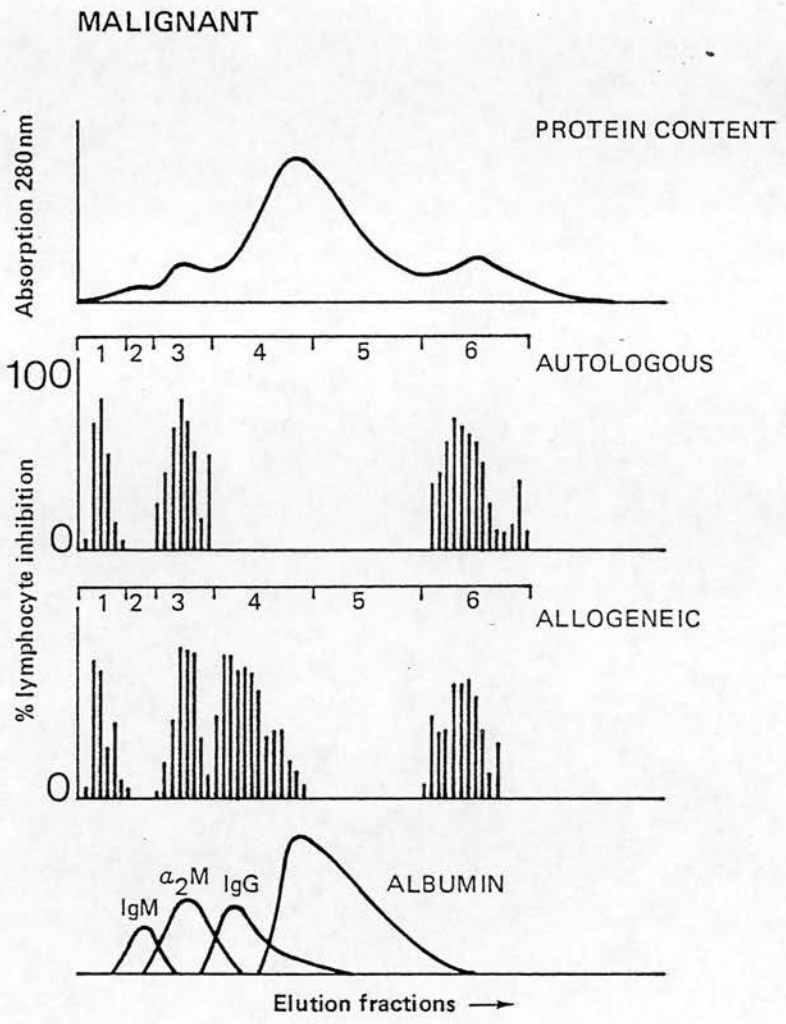


Figure 3.25 An example of the optical density, percentage inhibition of autologous and allogeneic lymphocytes and distribution of IgM, alpha 2 macroglobulin, IgG and albumin in the plasma fractions of a patient with gastric cancer

The brackets indicate the fractions which were pooled together to form the six regions or peaks (Section 3.6.2).

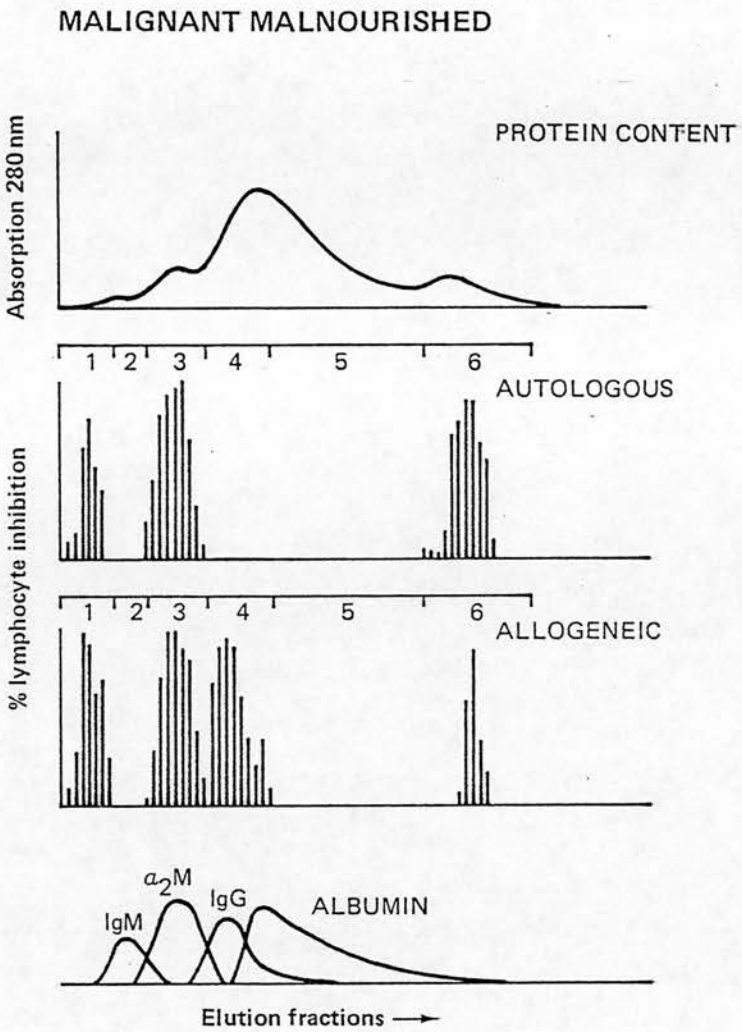


Figure 3.26 An example of the optical density, percentage inhibition of autologous and allogeneic lymphocytes and distribution of IgM, alpha 2 macroglobulin, IgG and albumin in the plasma fractions of a malnourished patient with a gastric carcinoma

The brackets indicate the fractions which were pooled together to form the six regions or peaks (Section 3.6.2).

of the histograms represents the percentage inhibition of one plasma fraction and SA was detected in only those fractions represented by a vertical line. The lowermost part of each Figure shows the relative positions of some plasma proteins in relation to the distribution of SA. Only a limited number of proteins have been shown for ease of presentation.

3.6.1 (b) Results

Plasma from normal subjects had SA against autologous and normal allogeneic lymphocytes concentrated in only a few fractions which formed a single region or peak corresponding to the distribution of the plasma protease inhibitor A2M (Figure 3.22). Similar results were obtained by fractionation of plasma from well nourished patients with benign disease (Figure 3.23).

A different pattern of results was found in the benign malnourished group (Figure 3.24). The optical density tracing was broadly similar to that of the benign well nourished except that the albumin peak was noticeably smaller. SA (autologous) was present in approximately twice as many fractions and was grouped into two distinct and quite obvious regions. One region corresponded almost exactly with the distribution of A2M, and the second region was placed after the albumin peak towards the low MW fractions. SA (allogeneic) was detected in more elution fractions which could be grouped into three obvious regions: one corresponding with A2M, another corresponding exactly with the low MW fractions which had SA against autologous lymphocytes, and another region which corresponded well with the distribution of IgG but no other protein.

Plasma fractions from well nourished cancer patients gave a different pattern of results (Figure 3.25). Optical density was similar to that of other well nourished subjects. Many fractions had SA against

autologous lymphocytes and formed three distinct regions: one with a MW greater than IgM, another corresponding to A2M and another at the low MW end of the elution range. This third region did not have the "bell" shape of the other regions, suggesting that it consisted of at least two subregions SA. SA against allogeneic cells was detected in a greater number of fractions and a fourth region of SA corresponding with the distribution of IgG was evident. The smaller MW region of SA (allogeneic) appeared to consist of at least two peaks in all patients.

The results of the malnourished cancer patients were broadly similar to the well nourished cancer group, except that the albumin peak was reduced in height. There were three peaks of SA against autologous lymphocytes, corresponding with the distribution of substances with MW above that of IgM, A2M and small MW substances. This third region (which was present in all samples from patients with cancer and those with benign disease and malnutrition) was broad, did not correspond with any protein detected by the anti-all human protein antibody and appeared to consist of several lymphocyte suppressive factors of differing MW. A fourth region of SA was identified by the use of allogeneic lymphocytes, this region corresponding with the distribution of IgG.

Representative examples of the percentage inhibition of autologous and allogeneic lymphocytes in plasma fractions from each subject group are given in Tables 3.13 - 3.17.

3.6.1 (c) Discussion

All of the plasma samples examined had a constant region of SA against both autologous and allogeneic lymphocytes that correspond to the location of A2M. Although the results do not prove that A2M is a

Table 3.13 Percentage inhibition of autologous and allogeneic lymphocytes. Plasma fractions from a normal subject

FRACTION NUMBER	PERCENTAGE LYMPHOCYTE INHIBITION	
	Autologous	Allogeneic
1 - 20 (void volume)	0	0
21 - 34	0	0
35	13.5	14.9
36	30.6	29.1
37	44.2	48.7
38	69.4	76.4
39	85.4	87.9
40	100	100
41	100	97.6
42	92.3	86.1
43	84.2	79.8
44	76.1	70.4
45	43.2	51.0
46	22.1	28.9
47	10.5	5.7
48	1.5	0
49	0	0
50 - 100	0	0

Table 3.14 Percentage inhibition of autologous and allogeneic lymphocytes. Plasma fractions from a patient with a benign gastric ulcer

FRACTION NUMBER	PERCENTAGE LYMPHOCYTE INHIBITION	
	Autologous	Allogeneic
1 - 20 (void volume)	0	0
21 - 31	0	0
32	7.5	4.6
33	19.8	16.4
34	39.5	39.0
35	79.6	85.1
36	100	100
37	100	100
38	94.6	91.2
39	72.2	66.7
40	45.0	38.9
41	26.4	17.3
42	8.6	15.3
43	0	5.2
44	0	1.8
45	0	0
46 - 100	0	0

Table 3.15 Percentage inhibition of autologous and allogeneic lymphocytes. Plasma fractions from a malnourished patient with a benign oesophageal stricture

FRACTION NUMBER	PERCENTAGE LYMPHOCYTE INHIBITION	
	Autologous	Allogeneic
1 - 20 (void volume)	0	0
21 - 32	0	0
33	0	2.9
34	3.3	9.5
35	4.4	65.6
36	48.6	89.1
37	71.0	100
38	100	69.3
39	77.6	58.4
40	42.6	58.0
41	34.4	18.3
42	20.8	15.2
43	4.9	10.9
44	0	52.5
45	0	65.0
46	0	60.6
47	0	73.0
48	0	78.1
49	0	55.5
50	0	18.3
51	0	6.2
52	0	0
53 - 81	0	0
82	4.4	0
83	28.3	7.8
84	53.4	29.7
85	76.0	75.4
86	98.7	93.4
87	65.7	53.9
88	54.4	48.5
89	73.0	56.9
90	69.0	58.2
91	49.5	37.2
92	22.5	20.6
93	5.5	4.4
94	2.9	0
95	0	0
96 -100	0	0

Table 3.16 Percentage inhibition of autologous and allogeneic lymphocytes. Plasma fractions from a patient with carcinoma of the stomach

FRACTION NUMBER	PERCENTAGE LYMPHOCYTE INHIBITION	
	Autologous	Allogeneic
1 - 22 (void volume)	0	0
23	0	0
24	44.2	10.2
25	66.4	54.5
26	77.7	78.2
27	69.5	92.3
28	77.1	78.3
29	40.4	49.4
30	9.2	9.4
31	0	0
30 - 33	0	0
34	2.2	4.6
35	12.2	37.4
36	24.6	60.3
37	57.4	92.8
38	100	100
39	100.6	88.0
40	85.9	89.3
41	65.6	80.1
42	21.7	79.4
43	17.5	73.3
44	0	65.6
45	0	33.6
51	13.5	55.0
52	5.8	62.7
53	0	78.0
54	0	71.7
55	0	61.0
56	0	90.2
57	0	86.3
58	0	54.7
59	0	42.5
60	0	26.7
61	0	19.6
62	0	31.2
63	0	19.7
64	0	14.5
65	0	9.7
66	0	2.5

Table 3.16 (continued)

FRACTION NUMBER	PERCENTAGE LYMPHOCYTE INHIBITION	
	Autologous	Allogeneic
67 - 90	0	0
91	4.7	16.7
92	20.7	39.5
93	34.0	34.5
94	89.0	75.6
95	78.9	83.2
96	67.9	52.5
97	23.4	34.0
98	20.5	9.1
99	0	0
100	0	0

Table 3.17 Percentage inhibition of autologous and allogeneic lymphocytes. Plasma fractions from a malnourished patient with gastric cancer

FRACTION NUMBER	PERCENTAGE LYMPHOCYTE INHIBITION	
	Autologous	Allogeneic
1 - 20 (void volume)	0	0
21	0	0
22	6.0	0
23	39.4	24.5
24	58.6	69.2
25	85.7	93.5
26	55.1	58.9
27	31.6	42.5
28	8.0	19.4
29	0	2.6
30 - 35	0	0
36	12.6	0
37	50.0	9.2
38	52.2	39.1
39	72.4	42.2
40	100	58.6
41	95.6	92.1
42	70.7	100
43	66.1	85.2

Table 3.17 (continued)

FRACTION NUMBER	PERCENTAGE LYMPHOCYTE INHIBITION	
	Autologous	Allogeneic
47	41.9	52.3
48	30.0	44.2
49	28.0	52.3
50	21.4	38.0
51	13.5	9.8
52	5.8	11.5
53	0	23.6
54	0	36.0
55	0	64.4
56	0	95.4
57	0	96.4
58	0	67.2
59	0	37.9
60	0	12.6
61	0	15.5
62	0	15.0
63	0	16.1
64	0	2.0
65	0	0
66 - 84	0	0
85	0	23.9
86	35.7	46.5
87	64.4	49.0
88	48.5	43.1
89	23.4	19.7
90	49.0	55.2
91	94.2	98.6
92	96.4	89.7
93	89.6	84.0
94	67.0	53.7
95	36.0	42.1
96	43.1	29.9
97	12.6	13.5
98	3.1	0
99	0	0
100	0	0

suppressive factor, they suggest strongly that this is the case. The results also suggest that A2M is a universal suppressive factor, if the results can be extrapolated from a small number of subjects to the population in general. Thirdly, it is evident that the suppressive nature of the region corresponding with A2M is non-specific as it inhibited the reactivity of both autologous and allogeneic lymphocytes.

The distribution of SA against autologous and allogeneic lymphocytes was the same in normal subjects and well nourished patients with benign disease - that is, a single region of SA corresponding with A2M. This finding might have been predicted from the observation that the SA of these two groups did not differ significantly (Sections 3.5.1 and 3.5.2).

Malnutrition was associated with an increase in the number of plasma fractions with SA. A small MW region with SA against autologous and allogeneic lymphocytes was present; the origin and nature of this region was uncertain and so further experiments were performed to elucidate its nature (Section 3.10). A peak of SA against allogeneic lymphocytes corresponded to the distribution of IgG (Section 3.7). The presence of additional fractions with SA was associated with co-existent nutritional impairment: although the two benign groups did not include patients with the same surgical conditions, only three of the 10 patients had a marked inflammatory component to their disease (diverticular disease (1) (well nourished), and ulcerative colitis (2) (malnourished)). However, the distribution and number of suppressive fractions was consistent within each patient group and was related to nutritional status rather than disease process. The presence of additional suppressive fractions is consistent with the finding of increased PSA in benign malnourished patients (Sections 3.5.1 and 3.5.2). However, the question of qualitative and quantitative

changes in naturally occurring suppressive factors as a result of disease states remains unanswered at this stage.

Cancer plasma had several regions of SA: the first consisted of substances of high MW (higher than that of IgM, 10^6 daltons), such as immune complexes or lipoproteins. Other suppressive regions coincided with A2M (autologous and allogeneic) and IgG (allogeneic only), and there was a broad band of suppressive fractions of small MW. These results are consistent with the earlier finding of a greatly increased PSA in cancer patients and indicate clearly that the SA was due to several factors in addition to A2M. The experiments suggest that SA was associated with specific plasma proteins.

3.6.2 POOLING OF PLASMA FRACTIONS

The SA identified by the above experiments was found in plasma fractions which were grouped naturally together (Figures 3.22 - 3.26). In order to verify the association of certain plasma constituents with SA, experiments were performed to quantify the SA of each suppressive region before and after removal of the plasma components. This necessitated pooling of plasma fractions to form specific regions of SA. Fractions were pooled on the basis of SA, and six regions were created from each plasma sample (Figures 3.25 and 3.26). The brackets around groups of fractions indicate which fractions were pooled together. The MW ranges and principal proteins within each of the six regions are shown in Table 3.18.

Generally, there was good agreement between the distributions of SA and plasma proteins, although proteins were not contained exclusively in just one region. For example, it is apparent in Figure 3.25 that most of the IgG was contained in region 4 but small amounts were present in the

Table 3.18 Molecular weight ranges and principal proteins in the pooled plasma fractions

REGION	MOLECULAR WEIGHT	PRINCIPAL PROTEINS
1	$> 10^6$	Immune complexes, beta lipoproteins
2	$10^6 - 8 \times 10^5$	IgM
3	$8 \times 10^5 - 2 \times 10^5$	Alpha-2-macroglobulin, alpha-1-lipoprotein, fibrinogen
4	$2 \times 10^5 - 9 \times 10^4$	IgG, alpha-1-lipoprotein, IgA, Transferrin
5	$9 \times 10^4 - 5 \times 10^4$	Albumin, pre-albumin, Transferrin, alpha-1-anti-trypsin, Caeruloplasmin
6	$< 5 \times 10^4$	Haptoglobin, acid-alpha-1-glycoprotein, low MW peptides

adjacent regions 3 and 5.

The volumes of each region of each experimental subject were measured and found to be almost identical, indicating a relatively constant pattern of SA for subjects within any one group.

3.6.3 QUANTIFICATION OF SUPPRESSIVE ACTIVITY IN THE SIX REGIONS

The SA of each of the six regions was measured against autologous and allogeneic lymphocytes so that the relative contributions of each region to the total PSA could be defined.

3.6.3 (a) Method

Throughout this work the first dilution of whole plasma to be assayed for SA was always 1 in 60. The measuring chamber of the cytopherometer had a volume of 3.0 ml and a 1 in 60 dilution of whole plasma was made by diluting 50 μl of plasma in 3.0 ml of Hank's BSS. However, 50 μl of each region would contain different amounts of plasma as the volumes of the six regions were different. To compare the SA of each region, it was necessary to standardise the dilution of each region by relating the dilution of each principal plasma protein to the volume of the region which contained the protein and not to the final dilution of the whole plasma sample. For example, IgM was contained in region 2 and so the dilution factor for IgM was the volume of region 2 divided by the volume of the original plasma sample (2.0 ml). The volume of each region which was made up to 3.0 ml with Hank's BSS was calculated by the formula:

$$VRR = VT \times DT \times DF$$

where VRR = Volume of the region required for SA determination

VT = Volume of the test sample (3.0 ml)

DT = Dilution of the test sample (1/60)

DF = Dilution factor (volume of region divided by volume of plasma, 2.0 ml)

For example, the mean volume of all second regions was 25.3 ml; therefore, the volume of region 2 required for SA assay was:

VRR = 3.0 x 1/60 x 25.3/2

= 0.6325 ml

= 632.5 μl

Therefore, 632.5 μl of region 2 was made up to 3.0 ml with Hank's BSS to produce a 1/60 dilution of "plasma equivalent". Similar calculations were performed for the other regions. The maximum volume of a region which could provide a 3.0 ml volume diluted 1 in 60 was 120 ml and, as some regions (especially region 6) exceeded 120 ml, it was necessary to concentrate them by ultrafiltration using Amicon Diaflo ultrafiltration membranes (Amicon, Massachusetts, U.S.A.). Filtration membranes were selected appropriately for each region on the basis of pore size so that all proteins within the region were retained by the membrane. The final volumes of the concentrated regions are given in Appendix 14. Dilutions of the volumes were made using the above formula to produce a 3.0 ml volume of 1 in 60 dilution suitable for assay by the TEEM test. Suppressive activity was expressed as the μl volume of the original plasma sample required to suppress the standard lymphocyte response by 50%, as described in Section 2.3.3.

3.6.3 (b) Results

The SA of each region from each of the 27 experimental subjects is shown in Table 3.19 and results are summarised in Table 3.20. Region 1

Table 3.19 Suppressive activities against autologous and allogeneic lymphocytes of each sample of each pooled region

Values are given as the equivalent volume of plasma (μl) required to suppress the lymphocyte response to PPD by 50%.

SUBJECT	N	BWN	BMN	MWN	MMN
Region 1 - Autologous					
1	-	-	-	24.0	32.4
2	-	-	-	19.8	20.4
3	-	-	-	17.7	34.6
4	-	-	-	42.4	45.5
5	-	-	-	39.8	39.6
6				36.1	39.2
Region 1 - Allogeneic					
1	-	-	-	24.0	37.7
2	-	-	-	38.2	80.0
3	-	-	-	18.4	18.7
4	-	-	-	36.3	45.0
5	-	-	-	36.2	35.8
6				32.5	37.0
Region 2 - No suppressive activity was detected					
Region 3 - Autologous					
1	35.7	34.0	18.8	3.05	5.32
2	35.0	39.4	35.1	2.8	5.4
3	35.4	46.5	7.9	2.31	2.11
4	34.3	42.3	9.5	1.41	2.7
5	30.5	39.8	12.6	1.18	8.6
6				2.26	3.05
Region 3 - Allogeneic					
1	38.1	35.7	15.8	1.12	4.8
2	38.0	40.9	35.8	2.06	1.22
3	37.2	44.2	4.2	2.09	2.0
4	38.0	39.6	4.37	1.08	1.64
5	35.5	40.6	4.5	1.12	2.35
6				2.22	2.18
Region 4 - Autologous. No suppressive activity was detected.					

Table 3.19 (continued)

Region 4 - Allogeneic					
1	-	-	76.0	4.5	19.0
2	-	-	0	34.8	30.6
3	-	-	33.2	18.0	35.0
4	-	-	29.0	35.1	35.0
5	-	-	36.0	18.3	57.1
6				20.0	37.1
Region 5 - No suppressive activity detected.					
Region 6 - Autologous					
1	-	-	63.2	10.7	16.7
2	-	-	75.6	15.2	34.4
3	-	-	31.0	19.6	24.3
4	-	-	35.3	19.8	37.5
5	-	-	30.2	37.1	29.3
6				40.0	42.8
Region 6 - Allogeneic					
1	-	-	74.2	9.9	16.6
2	-	-	73.8	20.2	37.4
3	-	-	35.4	18.6	8.5
4	-	-	30.1	9.1	17.0
5	-	-	36.2	34.4	33.0
6				19.6	49.0

N = Normal

BWN = Benign well nourished

BMN = Benign malnourished

MWN = Malignant well nourished

MMN = Malignant malnourished

Table 3.20 Suppressive activities (mean ± 1SD) against autologous lymphocytes in whole plasma and in each of the six regions of plasma

GROUP	n	SUPPRESSIVE ACTIVITY (μl plasma) (mean ± 1SD)						
		Whole plasma	Region					
			1	2	3	4	5	6
N	5	32.0 ±3.7	0	0	34.2 ±1.89	0	0	0
BWN	5	36.8 ±4.2	0	0	40.5 ±3.98	0	0	0
BMN	5	8.03 ±4.75	0	0	16.7 ± 9.8	0	0	47.1 ±18.7
MWN	6	1.59 ±1.28	29.9 ±9.81	0	2.16 ±0.67	0	0	23.7 ±10.9
MMN	6	2.25 ±1.09	35.2 ±7.83	0	4.53 ±2.21	0	0	30.8 ±8.6

N = Normal group

BWN = Benign well nourished group

BMN = Benign malnourished group

MWN = Malignant well nourished group

MMN = Malignant malnourished group

Table 3.20 (continued)

Suppressive activities (mean ± 1SD) against allogeneic lymphocytes in whole plasma and in each of the six regions of plasma

GROUP	n	SUPPRESSIVE ACTIVITY (μl plasma) (mean ± 1SD)						
		Whole plasma	Region					
			1	2	3	4	5	6
N	5	36.7 ±0.6	0	0	37.4 ±0.98	0	0	0
BWN	5	37.2 ±2.2	0	0	40.5 ±2.7	0	0	0
BMN	5	7.2 ±6.1	0	0	12.9 ±12.2	34.8 ±24.2	0	49.9 ±19.7
MWN	6	0.9 ±0.6	30.9 ±7.26	0	1.7 ±0.5	21.7 ±10.6	0	18.7 ±8.4
MMN	6	1.1 ±0.5	42.3 ±18.6	0	2.36 ±1.1	35.6 ±11.3	0	26.9 ±14.0

N = Normal group

BWN = Benign well nourished group

BMN = Benign malnourished group

MWN = Malignant well nourished group

MMN = Malignant malnourished group

contained SA (autologous and allogeneic) only in patients with malignant disease and there was no significant difference between the values of SA of the two groups with cancer. Region 2 was not suppressive in any subject. Region 3 was the only region found to be suppressive in all subjects, irrespective of the source of test lymphocytes. Region 4 was suppressive only in subjects with a significantly raised PSA (reduced μl volume of plasma) and only against allogeneic lymphocytes. No SA was detected in region 5. Region 6 had SA against autologous and allogeneic lymphocytes only in patients with elevated PSA. The value of SA (μl plasma) for any particular region was relatively constant for the subjects within each of the 5 experimental groups.

3.6.3 (c) Discussion

Region 3 contained the greatest amount of SA of any region, irrespective of whether subjects were healthy or had a benign or malignant disease. Comparison of the SA (μl plasma) of whole plasma and region 3 indicates that this region followed closely the pattern of SA in whole plasma and accounted for most of the SA in plasma. Therefore, as one might expect, the SA of region 3 was low (high μl volume of plasma) in normal subjects and well nourished benign patients, and significantly greater in benign malnourished patients and all patients with cancer. Thus, the region corresponding with the distribution of A2M was the principal non-specific lymphocyte suppressive factor in all subjects examined.

Regions 2 and 5 did not contain SA against autologous or allogeneic lymphocytes as determined by the TEEM test. Region 2 corresponded closely with the distribution of IgM, and region 5 contained several proteins including albumin, pre-albumin and transferrin. As this study was undertaken to examine immunosuppressive substances, the regions not having

SA (regions 2 and 5) were not examined any further.

Region 1, the region of greatest MW, was suppressive in all cancer patients. As SA was directed against autologous and allogeneic cells, the substances responsible for SA acted non-specifically. SA in region 1 was related in some way to the presence of a malignant tumour and was not a function of tumour type or stage.

Region 6 contained small MW substances and was suppressive against autologous and allogeneic lymphocytes in benign malnourished patients and cancer patients with and without nutritional impairment. It has already been suggested that this region may contain several lymphocyte suppressive factors, on the basis of the distribution of SA among fractions with different molecular weights. SA was non-specific. This suppressive region was present only in patients with tumours or in highly catabolic malnourished subjects, suggesting that the suppressive nature of the region was a result of tissue turnover or was due to products shed from actively dividing or catabolic cells.

Region 4 was suppressive in all subjects with a raised PSA (except one subject; Table 3.19) but was suppressive against allogeneic lymphocytes only. Thus, the suppressive factor(s) in region 4 could be a product of tissue breakdown or turnover (cf region 6) and was specific in its suppressive action in that suppression was not directed against host lymphocytes.

It has been shown in this section that some plasma regions with differing molecular weights contained lymphocyte SA, and that healthy subjects and those with benign or malignant disease differed in the amount of SA contained in these regions. Region 3 was the major non-specific suppressive region, accurately reflecting the total SA in whole plasma.

The SA of some regions coincided with the distribution of specific plasma proteins. Thus, further studies were performed to determine whether SA could be attributed to such substances and whether these factors differed quantitatively or qualitatively between the various subject groups.

3.7 STUDIES OF REGION 1

Region 1 consisted of the first 8 - 10 fractions eluted from the gel filtration column after the void volume, and contained substances with a minimum molecular weight of 10^6 daltons (Table 3.18). Region 1 contained proteins, as it had measurable optical density at 280 nm, and had SA against autologous and allogeneic lymphocytes in all cancer patients but no other experimental subject.

From the review of circulating non-specific immunosuppressive factors associated with malignancy (Section 1.5.7) it was thought that immune complexes (IC) could be responsible for some or all of the SA in region 1. IC have heavy MW and so would be located in region 1, and as they have non-specific SA (Section 1.5.7(a)) they would inhibit both autologous and allogeneic lymphocytes. Beta - lipoproteins would be present in region 1 also but there was no reason for lipoproteins to be suppressive in cancer patients but not other subjects. Therefore, it was decided to investigate region 1 by firstly separating IC from the region, and secondly measuring SA after depletion of IC.

3.7.1 METHOD

The polyethylene glycol (PEG) method[352] was used to precipitate IC (Section 2.8). Three PEG dilutions were used - 5%, 10% and 20% - as recommended by Creighton[352], to increase the sensitivity of the technique. SA was measured using allogeneic lymphocytes because autologous

cells were not available in some cases when samples were tested. A 1 in 30 dilution was the first dilution used for measuring SA and subsequent dilutions were 1 in 60, 1 in 120 and so on.

3.7.2 RESULTS

Precipitates of IC were obtained from all region 1 samples of patients with malignant disease but not in any sample from normal subjects or patients with benign disease. IC precipitates were seen as small white pellets or thin white rims at the bottom of the conical glass centrifuge tubes in which PEG precipitation was performed. There was no visible quantitative difference in the precipitates obtained with the three PEG dilutions.

The SA of untreated region 1, the precipitates obtained with 5% PEG and the supernatants were measured using allogeneic lymphocytes (Table 3.21). The value for each SA measurement was the mean of the values obtained from duplicate samples. The percentage recovery of SA in the precipitates was calculated by dividing the SA of region 1 by the SA of the precipitate. As no SA had been detected previously in the region 1 of normal subjects and benign patients, SA measurements of these regions were not repeated.

SA activity was detected in all precipitates (with 5% PEG), and in nearly all patients the SA of the precipitate corresponded closely with SA in the untreated region 1. No supernatant contained SA at a 1 in 30 dilution, indicating that IC accounted for all of the SA in region 1.

3.7.3 CONTAMINATION BY FREE IMMUNOGLOBULIN

The precautions taken to avoid contamination of region 1 by free

Table 3.21 Suppressive activities (allogeneic) of region 1, precipitates and supernatants

SUBJECT	SUPPRESSIVE ACTIVITY (μl plasma)			
Malignant well nourished	Region 1	Precipitate with 5% PEG	Supernatant	% recovery of SA in precipitate
1	24.0	76.0	ND	32%
2	38.2	38.1	ND	100%
3	18.4	20.2	ND	91%
4	36.3	40.1	ND	91%
5	36.2	38.9	ND	93%
6	32.5	43.9	ND	93%
Malignant malnourished				
1	37.7	38.0	ND	99%
2	80.0	66.2	ND	120%
3	18.7	19.8	ND	94%
4	45.0	40.2	ND	111%
5	35.8	39.0	ND	92%
6	37.0	38.7	ND	96%

SA = Suppressive activity

ND = Not detected

immunoglobulins were stated in Section 2.8. While region 1 may have been contaminated slightly by IgM (contained predominantly in region 2) it is highly unlikely that any free IgG would be found in region 1. IgG (MW = 150,000 daltons) was collected in region 4, the beginning of which was separated from the last fraction of region 1 by at least 20 elution fractions. Also, region 1 was concentrated by ultrafiltration using a filter which retained only substances of MW greater than 300,000 daltons. Thus, any IgG found within region 1 could not have been due to contamination by free IgG, and so comparison of IgG levels within the untreated region 1 and within the precipitate would provide a means of assessing the efficiency of the PEG method.

3.7.3 (a) Method

Each region 1 and corresponding precipitate (with 20% PEG) of the 12 cancer patients was titrated with dilute HCl until a pH of 4.0 was reached. Samples were acidified to dissociate the antigen-antibody complexes[359]. Precipitates obtained with 20% PEG were used because 20% PEG was more likely to have precipitated free immunoglobulin than the other two dilutions[352]. IgG concentrations were measured by single radial immunodiffusion (Section 2.9). A 10 μl volume of each sample was used in the wells of the immunodiffusion plates.

3.7.3 (b) Results

There was a strong and highly significant correlation between IgG concentrations in untreated region 1 and in precipitates of region 1 (Figure 3.27), indicating that the PEG precipitation technique was highly efficient in precipitating IC which contained IgG. The percentage of IgG in the untreated region recovered in the precipitate was between 58% -

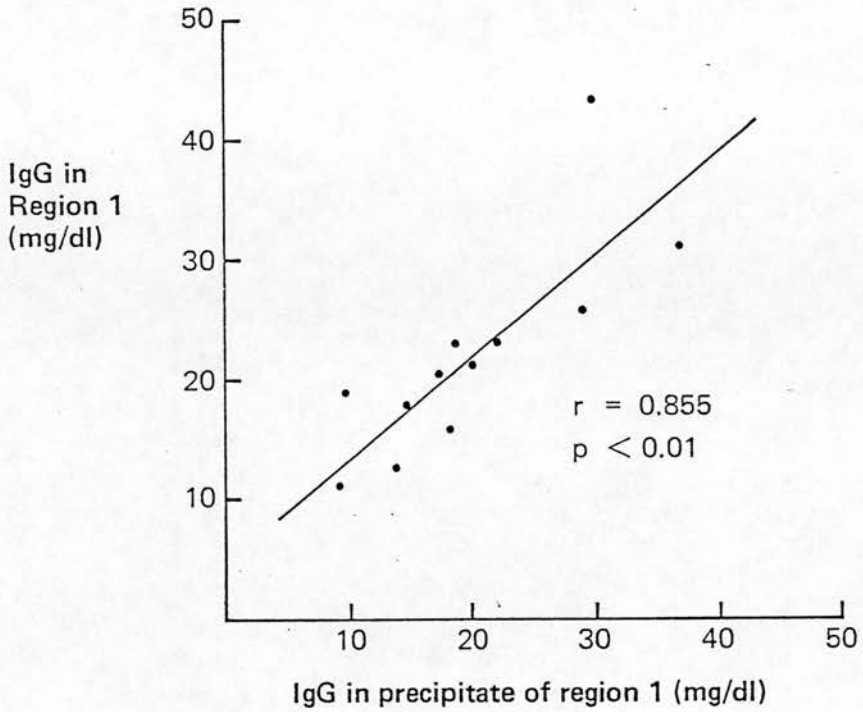


Figure 3.27 The correlation between the concentrations of IgG in original region 1 samples and in precipitates of region 1

121%, with a median of 93.5%. The percentage recovery was greater than 100% in four cases, presumably due to experimental error.

3.7.4 DISCUSSION

SA was found in region 1 samples of cancer patients only, and precipitation of IC with 5% PEG removed all SA from the samples. Also, the SA of precipitates approximated to that of the regions before IC were precipitated. Measurements of IgG in the untreated region and in the precipitates showed good correlation, and the overall recovery rate of IgG in precipitates was 93%. Thus, the method of precipitating IC was effective - IgG in the precipitates could have come only from soluble IC of region 1. Thus, IC were present in patients with malignant disease (irrespective of nutritional status), and accounted for some of the SA detected in cancer plasma. The non-specific nature of the SA of IC has been demonstrated by the use of autologous and allogeneic cells. From the results obtained in Section 3.6.3, it is clear that there were other suppressive factors in the plasma of cancer patients; IC contribute in part to the increased PSA of cancer patients but not in healthy subjects or patients with benign disease.

3.8 STUDIES OF REGION 3

Region 3 consisted of fractions with MW between 8×10^5 and 2×10^5 daltons and was the only region to exhibit SA against autologous and allogeneic lymphocytes in every experimental subject. The amount of SA in region 3 was relatively constant for subjects within each group; normal and well nourished subjects with benign disease had relatively little SA in region 3, whereas well nourished and malnourished cancer patients had considerable SA associated with this region; the malnourished benign group

were between these two extremes. The SA in region 3 closely paralleled that of whole plasma and accounted for the majority of SA of whole plasma in all subjects. Studies of the eluted plasma fractions with fused rocket immuno-electrophoresis suggested strongly that the protease inhibitor alpha 2 macroglobulin (A2M) was the principal plasma protein in fractions pooled to form region 3.

To demonstrate that A2M was responsible for the SA in region 3, A2M was removed from the region by immuno-specific affinity chromatography (Section 2.7). SA and A2M concentrations were measured in samples of (i) whole plasma, (ii) untreated region 3, (iii) elutions from the affinity chromatography columns, and (iv) the A2M which bound to affinity chromatography columns.

3.8.1 METHOD

Two affinity chromatography columns were constructed (Section 2.7.1), one with anti-A2M conjugated to the column matrix and the other conjugated with normal rabbit serum to act as a control to detect non-specific binding of proteins. SA was measured against allogeneic lymphocytes only, as autologous cells were not always obtainable when these studies were performed.

The volumes of each region 3 applied to the columns were equivalent to 1.0 ml of plasma, as calculated from the final volumes of region 3 (Appendix 14). If this volume was less than 2.0 ml it was made up to 2.0 ml with Stavitsky's PBS. A2M was removed from the anti-A2M column by washing the column with 20 ml 3M sodium thiocyanate. The thiocyanate solution was strongly ionic and eluates containing sodium thiocyanate could not be assayed for SA in the TEEM test. Therefore, thiocyanate was removed

by dialysis by placing the thiocyanate eluate in a bag of Visking tubing which was then sealed; dialysis was performed against Hank's BSS (500 ml for 10 dialysis bags) for four hours and this was sufficient to remove the sodium thiocyanate.

3.8.1 (a) Alpha 2 macroglobulin concentration

A2M concentrations were measured by single rocket immuno-electrophoresis (Section 2.6). Plasma dilutions were 1 in 10 and 1 in 20 for A2M measurements of whole plasma. Dilutions of the column elutions were 1 in 20, a volume equivalent to 1.0 ml of plasma having been applied to the column, which was washed with 20 ml of Stavitsky's PBS per sample. Dilutions of the standard A2M antigen were 1 in 10, 1 in 20, 1 in 40 and 1 in 60 for single rocket immuno-electrophoresis plates measuring A2M in column elutions, and 1 in 5, 1 in 10, 1 in 20 and 1 in 50 for plates measuring plasma levels of A2M (Appendix 10).

3.8.1 (b) Suppressive activity

The volume of each region 3 used for assay of SA was the volume which contained the equivalent of 50 μl of plasma, made up to 3.0 ml with Hank's BSS, producing a 1 in 60 dilution as the first dilution for the TEEM test and allowing serial dilutions (1/120, 1/240 and so on) to be made in the usual manner. The elution of each sample of region 3 from the affinity columns was a 1 in 20 dilution of plasma. It was anticipated that removal of A2M by affinity chromatography would reduce the SA in the column elutions and so lower dilutions were used to detect SA. The first dilution was 1 in 20. A 5.0 ml aliquot of the elution was used to dialyse out the thiocyanate solution.

3.8.2 RESULTS

3.8.2 (a) Alpha-2-macroglobulin concentration

There was no significant difference in the concentrations of A2M in plasma or in region 3 between the five experimental groups (Tables 3.22 and 3.23). The range of A2M concentrations in whole plasma was wide, in keeping with the wide variation of A2M in normal subjects (Section 1.5.8). The mean percentage recovery of whole plasma A2M in region 3 was 85%; the apparent loss of some 15% represents losses due to diluting procedures, the fractionation method and possible error in the technique of measuring A2M concentration. Another source of error was the pooling of column fractions containing A2M into regions 2 and 4; it can be seen from Figures 3.25 and 3.26 that proteins were distributed over a range of several fractions, and it was inevitable that some A2M at the extremes of its distribution curve would be included in the two adjacent regions.

The recovery rate of A2M from the control affinity chromatography column was consistently in the order of 90-95%. A slight loss of A2M occurred with samples from all groups and may have been due to non-specific binding to the column.

No A2M was recovered in any elution obtained from the column conjugated with anti-A2M antibody (Table 3.23). Using dilutions of standard A2M antigen the lowest concentration of A2M which could be detected by single rocket immuno-electrophoresis in our laboratory was 0.5 mg/100 ml. Therefore, as A2M was not detected in the elutions, it was highly unlikely that any significant quantity was present.

3.8.2 (b) Suppressive activity

No SA was detected in region 3 samples after passage through the anti-A2M affinity chromatography columns, whereas SA in elutions from the

Table 3.22 Alpha-2-macroglobulin concentrations in plasma and in region 3

SUBJECT		ALPHA 2 MACROGLOBULIN CONCENTRATION (mg/100ml)		
		Plasma	Region 3 -	% recovery in region 3
Normal	1	178	125	70
	2	115	65	56
	3	200	184	92
	4	300	274	91
	5	213	189	88
Benign well nourished				
	1	225	201	89
	2	307	269	88
	3	128	120	94
	4	214	150	70
	5	198	171	86
Benign malnourished				
	1	145	120	83
	2	317	275	86
	3	141	97	69
	4	230	200	87
	5	345	248	72
Malignant well nourished				
	1	245	231	94
	2	180	164	91
	3	150	119	79
	4	201	152	76
	5	280	215	77
	6	160	134	84
Malignant malnourished				
	1	155	138	89
	2	269	249	93
	3	301	300	99
	4	197	127	65
	5	215	179	83
	6	237	180	76

Alpha 2 macroglobulin was not detected in elutions from the anti-alpha 2 macroglobulin affinity chromatography columns

Table 3.23 Alpha-2-macroglobulin concentrations (mean ± 1SD) in plasma and in region 3 before and after anti-alpha-2-macroglobulin affinity chromatography

GROUP	n	ALPHA 2 MACROGLOBULIN CONCENTRATION (mg/100ml) (mean ± 1SD)			
		Plasma	Region 3	Elution from anti-alpha 2 column	Thiocyanated eluate
N	5	201.2 ±59.8	167.4 ±69.8	ND	ND
BWM	5	214.4 ±57.3	182.2 ±50.8	ND	ND
BMN	5	235.6 ±84.6	188.0 ±69.5	ND	ND
MWN	6	202.6 ±46.3	169.2 ±40.8	ND	D
MMN	6	229.0 ±47.5	195.6 ±61.2	ND	D

N = Normal

BWN = Benign well nourished

BMN = Benign malnourished

MWN = Malignant well nourished

MMN = Malignant malnourished

ND = Not detected

D = Detected in very small quantities in some samples only

control column closely resembled that of untreated region 3 (Tables 3.24 and 3.25). SA was detected in samples eluted from the anti-A2M column with sodium thiocyanate; these samples were dialysed to remove the thiocyanate before SA was measured. The values of SA in 'dialysed thiocyanate' eluates resembled closely those of untreated region 3 and the recovery of SA in these samples for the five experimental groups was 80.7% - 98.6%. Thus, it is clear that the use of thiocyanate to remove A2M resulted simply in uncoupling of antigen from antibody and not denaturation of the A2M molecule.

3.8.3 THE BIOLOGICAL BEHAVIOUR OF ALPHA-2-MACROGLOBULIN

The experiments reported in Section 3.8 show that (i) the amounts of A2M in region 3 of each experimental group were very similar, and differences between the five groups with respect to A2M concentrations were not statistically significant (Table 3.22), (ii) the amount of A2M in region 3 closely matched the A2M concentration in whole plasma for all samples examined (Table 3.22) and (iii) the SA of region 3 was associated entirely with A2M, as removal of A2M removed all SA from the region; A2M was recovered from immunospecific anti-A2M affinity chromatography columns and was associated with SA, the levels of which were very close to those in the untreated region 3 (Table 3.24). The amount of SA associated with region 3 depended on the nature of the experimental group (Table 3.20): SA was low in normal subjects and well nourished benign patients, increased in malnourished patients with benign disease and relatively very high in both groups of cancer patients. As the SA in region 3 of all five subject groups was associated entirely with A2M, it is clear that the biological behaviour of A2M in the various subjects was remarkably different: in normal subjects A2M had little SA whereas A2M was highly suppressive in

Table 3.24 Suppressive activity against allogeneic lymphocytes of region 3 before and after affinity chromatography

SUBJECT	SUPPRESSIVE ACTIVITY (μl plasma)			
	Region 3	Column elutions		Dialysed thiocyanate eluate
		Control	Anti-A2M	
Normal				
1	38.1	37.8	0	39.1
2	38.0	39.0	0	39.0
3	37.2	39.5	0	38.9
4	38.0	38.2	0	38.5
5	35.5	37.0	0	36.3
BWN				
1	35.7	37.3	0	54.2
2	40.9	39.6	0	41.6
3	44.2	46.6	0	45.0
4	39.6	39.3	0	42.3
5	40.6	46.6	0	41.7
BMN				
1	15.8	20.9	0	14.5
2	35.8	34.9	0	37.2
3	4.2	4.5	0	4.6
4	4.4	4.9	0	4.7
5	4.5	4.8	0	4.6
MWN				
1	1.12	1.90	0	1.15
2	2.06	2.10	0	2.60
3	2.09	2.41	0	2.31
4	1.08	1.00	0	2.30
5	1.12	1.32	0	1.22
6	2.22	2.35	0	2.38
MMN				
1	4.80	4.95	0	4.88
2	1.22	1.38	0	1.17
3	2.00	2.33	0	2.21
4	1.64	1.76	0	1.87
5	2.35	2.44	0	2.50
6	2.18	2.27	0	2.22

Table 3.25 Summary of suppressive activities against allogeneic lymphocytes of region 3 before and after affinity chromatography

GROUP	SUPPRESSIVE ACTIVITY (μl plasma) (mean ± 1SD)			
	Region 3	Elution from anti-A2M column	Dialysed thiocyanate elution	(% recovery)
Normal	37.36 ±0.98	0	38.36 ±1.05	(97.3%)
Benign well nourished (BWN)	40.2 ±2.73	0	44.9 ±4.78	(89.0%)
Benign malnourished (BMN)	12.94 ±12.25	0	13.12 ±12.63	(98.6%)
Malignant well nourished (MWN)	1.61 ±0.51	0	1.99 ±0.58	(80.7%)
Malignant malnourished (MMN)	2.36 ±1.14	0	2.47 ±1.15	(95.3%)

cancer patients. The differences in behaviour can be quantified by calculating the amount of A2M from each region 3 of each patient required to inhibit a standardised allogeneic lymphocyte response, by 50%.

Relatively large amounts of A2M in normal subjects (44.3-176.0 μg) and well nourished benign patients (45.6 - 226.3-μg) were required to produce 50% inhibition (Table 3.26). Much smaller amounts of A2M from malnourished benign patients (8.5 - 183.0 μg), and even smaller quantities of A2M from cancer patients (well nourished: 2.4 - 4.8 μg; malnourished: 2.7 - 22.3 μg) were required for the same degree of suppression.

3.8.4 DISCUSSION

Region 3 was the only one of the six regions of plasma to exhibit SA against both autologous and allogeneic lymphocytes in all subjects, an observation which suggests that the factor responsible for SA in this region was non-specific and universal. The SA of region 3 accounted for all (normal, benign well nourished) or most (benign malnourished, malignant) of the SA of whole plasma, indicating that the factor responsible was the most important naturally occurring lymphocyte suppressive factor as determined by the TEEM test assay in these patients. It has been shown clearly that the SA of region 3 is due entirely to A2M. As levels of A2M in region 3 were similar in all experimental groups, but levels of SA differed greatly, it is evident that the biological behaviour of A2M was dramatically different in some of the experimental groups.

The association of a highly suppressive A2M with malignant disease and tissue catabolism indicates that A2M has an important role in immunoregulation and the immunosuppression which accompanies some disease states. The present study has shown for the first time that immune

Table 3.26 Quantities of alpha-2-macroglobulin (A2M) causing 50% inhibition of allogeneic lymphocyte responses to PPD antigen

SUBJECT	DILUTION OF REGION 3 CAUSING 50% INHIBITION	AMOUNT OF A2M IN REGION 3 (mg)	AMOUNT OF A2M CAUSING 50% INHIBITION (μg)
Normal			
1	1 in 78	6.5	82.5
2	79	3.5	44.3
3	81	4.4	54.3
4	79	13.9	176.0
5	84	10.0	118.0
BWN			
1	1 in 77	9.6	124.8
2	73	16.6	226.3
3	68	3.1	45.6
4	76	5.9	77.8
5	74	8.6	116.3
BMN			
1	1 in 190	3.7	19.4
2	84	15.4	183.0
3	714	6.1	8.5
4	682	12.4	18.1
5	667	16.6	25.0
MWN			
1	1 in 2678	10.4	3.8
2	1456	6.8	4.7
3	1376	6.6	4.8
4	2654	9.1	3.4
5	2678	12.6	4.7
6	1351	3.2	2.4
MMN			
1	1 in 625	4.0	6.4
2	555	12.4	22.3
3	1470	10.5	7.1
4	1829	4.9	2.7
5	1276	11.1	8.6
6	1382	7.7	5.6

depression can be achieved by a change in the biological behaviour of the substance and not an increase in its absolute blood level. This theme will be discussed further in the context of additional experimental results (Chapter 6).

3.9 STUDIES OF REGION 4

Only region 4 obtained from cancer patients and from benign malnourished patients contained SA which was directed against allogeneic but not autologous lymphocytes. The MW range of region 4 was 2×10^5 to 9×10^4 daltons, and included alpha-1-lipoprotein, caeruloplasmin, lactate dehydrogenase, IgG, IgA and transferrin. The observation that region 4 SA was directed against only allogeneic cells suggested that it was due to immunologically specific molecules. Therefore, the first experiment with this region aimed to establish an association between the major immunoglobulin in this region (IgG) and SA.

3.9.1 METHOD

Immunospecific affinity chromatography was used to deplete region 4 of IgG by the method described in Section 2.7. A control column with adhered rabbit serum was also used. A volume of each region 4 equivalent to 1.0 ml of plasma was applied to the columns. IgG was eluted from the columns with 20 ml of 3M sodium thiocyanate which was removed from the eluate by dialysis against Hank's BSS (Section 3.8.1), and the eluate was assayed for SA. The SA of IgG-depleted region 4 was assayed against allogeneic lymphocytes. SA in region 4 was detected only in cancer patients and benign malnourished patients and so further studies were performed only with these experimental groups.

3.9.2 RESULTS

The SA of region 4 accounted for only a small proportion of the SA of whole plasma (Table 3.27). The SA of the eluate from the control column closely approximated that of untreated region 4 indicating that minimal non-specific binding occurred. Virtually no SA was detected in the eluate from the anti-IgG column: in five patients a 'trace' of SA was detected but no activity was found in the other 12 patients. The dilution of this eluate was 1 in 20, as opposed to the usual 1 in 60 dilution. SA was detected in all dialysed eluates obtained after washing the column with sodium thiocyanate and the percentage recovery of activity was generally high (91 - 93%).

3.9.3 DISCUSSION

Depletion of IgG from region 4 resulted in loss of virtually all lymphocyte SA which was recovered later in the eluate removed from the column by 3M sodium thiocyanate. The finding that IgG was suppressive is consistent with the finding that region 4 did not suppress autologous lymphocytes.

The detection of traces of SA in the regions of some patients after removal of IgG suggests the presence of other suppressive substances. The most likely explanation is that A2M (found chiefly in region 3) contaminated the region. The distributions of A2M and IgG among gel filtration fractions overlapped to some extent (Figures 3.23 - 3.26) and contamination of region 4 with small amounts of A2M was inevitable.

The relevance of this suppressive region to the overall immune depression so frequently observed in cancer and malnourished patients is open to question because SA was directed against allogeneic lymphocytes only. It is reasonable to conclude that region 4 (IgG) does not contribute

Table 3.27 Suppressive activity (allogeneic) of region 4 before and after affinity chromatography

SUBJECT	SUPPRESSIVE ACTIVITY (μl plasma)					
	Whole plasma	Region 4	Eluate from		Dialysed thiocyanate eluate	
			Control column	Anti-IgG column	SA	% recovery
BMN						
1	16.5	74.5	71.6	0	77.0	
2	18.7	70.4	74.5	0	78.0	
3	2.2	33.4	32.9	0	35.6	
4	3.9	29.1	33.0	0	34.0	
5	2.7	36.0	34.1	0	37.6	
MEAN±1SD	8.8±7.2	48.7±19.5	49.2±19.4	0	52.4±20.5	93%
MWN						
1	0.68	4.5	5.2	T	5.0	
2	2.15	34.8	34.2	0	38.3	
3	0.94	18.2	18.9	T	20.2	
4	0.52	35.1	34.9	0	37.9	
5	0.28	18.3	18.0	T	20.2	
6	0.97	20.0	19.6	T	21.7	
MEAN±1SD	0.92±0.6	21.8±10.6	21.8±10.2	0	23.9±11.5	91%
MMN						
1	1.65	19.0	19.2	0	26.5	
2	0.57	30.6	31.0	0	31.4	
3	0.54	35.0	35.3	0	37.3	
4	1.84	35.0	36.0	0	39.0	
5	1.42	57.1	57.5	0	58.5	
6	0.97	37.1	36.9	T	38.5	
MEAN±1SD	1.2±0.5	35.6±11.3	35.9±11.3	0	38.5±9.9	92%

BMN = Benign malnourished
MWN = Malignant well nourished
MMN = Malignant malnourished
T = Trace amount of suppressive activity detected
SA = Suppressive Activity

to host lymphocyte suppression. The possible role of IgG in the immunological escape of tumours has been reviewed in Section 1.5.5 and, while it is likely that IgG does contribute to tumour advancement in some situations, it seems unlikely from the present results that the mechanism is by depression of host lymphocyte reactivity.

It is of interest to speculate upon the origin of the 'suppressive IgG' in malnourished patients and the fact that IgG was present in normal and well nourished subjects and yet it did not suppress allogeneic lymphocytes. It is possible that the former group had subclinical infections and that the IgG represented a recent or current humoral response. This finding could alternatively reflect simply levels of IgG in plasma: IgG may be elevated in malnourished subjects (Section 1.6.1).

3.10 STUDIES OF REGION 6

Suppressive activity against autologous and allogeneic lymphocytes was detected in region 6 in all of the 12 patients with malignant disease and in the five malnourished patients with benign diseases. Region 6 contained substances with MW less than 5×10^4 daltons, such as trypsin, amylase, haptoglobin, alpha-1-antitrypsin, and acidic alpha-1-glycoprotein (Figures 3.22 - 3.26). Also, small MW immunoglobulin fragments or subunits may have been present in this fraction of plasma. The possible role of immunoglobulin fragments in the immunological escape of tumours has been mentioned in Section 1.5.7(d) and it was decided to investigate the possibility that Fc subunits of IgG contributed to the lymphocyte SA of plasma.

3.10.1 METHOD

Only regions (region 6) exhibiting SA were studied, Fc fragments of

IgG (IgG-Fc) were removed by immunospecific affinity chromatography. An affinity chromatography column was prepared bearing antibody to the Fc fraction of IgG, as described in Section 2.7. A control column was used to detect non-specific antigen binding. The volume of each region 6 applied to the column was the volume of the region derived from 1.0 ml of the original plasma sample. SA against allogeneic lymphocytes was measured in untreated region 6, in the eluate obtained after incubation with the affinity chromatography columns and in the eluate removed from the columns by 3M sodium thiocyanate (Section 2.7.2). The percentage of SA lost as a result of depleting the samples of Fc fragments was calculated by comparing the SA of paired eluates obtained from the control and anti-Fc columns.

3.10.2 RESULTS

Following depletion of Ig-Fc, SA fell in all samples by 1 - 69% (Table 3.28). However, SA was detected in the eluate from the anti-Fc column, indicating that Ig-Fc accounted for only a relatively small proportion of the SA of the region.

In five patients (numbers 1, 2 and 5 benign malnourished, and number 1 malignant well nourished, and number 2 malignant malnourished) depletion of Ig-Fc resulted in reduction of SA by only a few percent, suggesting that the amount of Ig-Fc in the region was very small. Previous studies in the laboratory (not reported here) indicated that the experimental error of the technique was about 4 - 5%, and so it is conceivable that there were no Fc fragments within this region in some subjects. The average reductions in SA following Ig-Fc depletion for the benign malnourished and well nourished and malnourished cancer patients were 16.8%, 29.5% and 35% respectively. Suppressive activity was difficult

Table 3.28 Suppressive activity against allogeneic lymphocytes of region 6 before and after affinity chromatography

SUBJECT	SUPPRESSIVE ACTIVITY (μl plasma)				
	Region 6	Eluate from		% of SA lost by IgG/Fc depletion	Dialysed thiocyanate eluate
		Control column	Anti-IgG/Fc column		
BMN					
1	74.2	74.6	75.7	2%	ND
2	73.8	74.2	78.4	5%	T
3	35.4	43.0	53.2	18%	ND
4	30.1	31.1	62.5	50%	ND
5	36.2	36.0	39.6	9%	ND
MEAN±1SD	49.9±19.7	50.2±19.8	59.8±16.1	16.8%	
MWN					
1	9.9	9.8	10.4	6%	T
2	20.2	20.8	37.6	47%	ND
3	18.6	19.2	38.7	50%	ND
4	9.1	9.8	11.8	17%	ND
5	34.4	36.7	43.8	16%	ND
6	19.6	21.8	36.9	41%	ND
MEAN±1SD	18.6±8.3	19.6±9.0	29.9±13.4	29.5%	
MMN					
1	16.6	19.3	53.5	64%	T
2	37.4	36.1	35.8	1%	ND
3	8.5	10.2	33.2	69%	ND
4	17.0	19.5	36.8	47%	ND
5	33.0	53.2	40.6	13%	ND
6	49.0	49.6	59.9	17%	ND
MEAN±1SD	26.9±14.0	28.3±13.2	43.3±9.9	35%	

BMN = Benign malnourished
MWN = Malignant well nourished
MMN = Malignant malnourished

ND = Not detected
T = Trace amount of suppressive activity detected
SA = Suppressive activity

to detect in the dialysed eluate removed from the anti-Fc column with sodium thiocyanate and traces of activity were detected in only three subjects. It is likely that suppressive substances were either dialysed out with the sodium thiocyanate or were degraded by this strongly ionic salt with subsequent loss of SA.

3.10.3 DISCUSSION

The experiment indicates that the SA of region 6 was due only in part to Fc fragments of IgG and that other substances with suppressive effects on allogeneic lymphocytes were present in this low MW region. The importance of Ig-Fc in lymphocyte suppression appeared to differ between benign and malignant patients. Firstly, three of five benign malnourished subjects had only marginal reductions of SA after Ig-Fc depletion, indicating that either Ig-Fc made very little contribution to SA or there was very little Ig-Fc in the region. However, similar findings occurred in only two of twelve malignant patients (17%). Secondly, the mean % reduction of SA after IgG-Fc depletion increased in a step wise fashion, indicating that Ig-Fc made the largest contribution to SA in the malnourished malignant group and the least in the malnourished benign group. These findings could be interpreted as Ig-Fc having an additive effect in malnutrition and malignancy such that these fragments were most abundant when the two conditions co-existed.

The potential importance of Fc fragments has been mentioned in the literature review (Section 1.5.7(b)) and their relevance to mechanisms of tumour escape will be discussed in Chapter 6.

The importance of the lymphocyte SA of Fc fragments to all patients with malignant disease and malnutrition secondary to benign disease is questioned by the inconsistent results in the small number of patients

studied here. It seems likely that, in some cancer patients at least, Fc subunits of IgG do have an important inhibitory effect on lymphocyte reactivity. The origin of the Ig from which the fragments were derived is unknown. Unfortunately, time did not allow the author to undertake further studies of the lymphocyte SA of other low MW substances in the region, although studies were undertaken subsequently in the laboratory by others.

3.11 SUMMARY

The experimental results presented in this chapter show that in 136 subjects who were normal volunteers or patients coming to surgery for benign or malignant disease:

(i) *in vitro* lymphocyte reactivity to a recall antigen (PPD) and three mitogens (PHA, Con A and PWM) was reduced in patients with malignant disease and to a lesser extent in patients with benign disease, compared with responses of healthy individuals.

(ii) lymphocyte responses correlated significantly with all parameters of nutritional assessment (weight for height, weight loss, TSF thickness, AMC and serum albumin) in patients with benign conditions; lymphocyte responses to Con A and PWM did not correlate with nutritional parameters in patients with malignant disease although responses to PPD and PHA correlated significantly. Generally, the sensitivity of lymphocyte reactivity was low in patients with malignancy.

(iii) in the absence of nutritional impairment lymphocyte reactivity in patients with benign conditions did not differ significantly from that of normal subjects whereas lymphocyte reactivity was reduced in patients who were adequately nourished but had cancer.

(iv) plasma from all subjects had the ability to suppress the *in vitro*

reactivity of autologous and allogeneic lymphocytes to PPD.

(v) the SA of plasma was significantly increased in patients who were malnourished because of benign disease, and was much greater in patients with cancer, irrespective of nutritional status.

(vi) there was no clearly defined relationship between the increase in plasma SA and the extent or tissue of origin of the malignant disease.

Experiments were performed with plasma obtained from five healthy volunteers and 22 patients with either benign or malignant diseases:

(vii) within each subject group a consistent pattern of plasma-related lymphocyte suppression emerged such that fractions of plasma could be grouped together to form regions of SA.

(viii) in healthy subjects SA was relatively low and was due entirely to A2M which had SA against autologous and allogeneic lymphocytes.

(ix) the pattern of SA in well nourished patients with benign disease was the same as in healthy subjects.

(x) SA in malnourished benign patients was associated mainly with A2M but also with a small MW fraction, and the IgG of these patients had a suppressive effect on allogeneic lymphocytes.

(xi) SA in all of the cancer patients was associated mainly with A2M but also with immune complexes, Fc fragments of IgG, small MW substances and IgG (allogeneic cells).

(xii) the plasma concentrations of A2M in each experimental groups did not differ significantly whereas the SA associated with A2M did, indicating that the biological behaviour of the molecule was different in groups with high SA.

CHAPTER 4

IMMUNOLOGICAL STUDIES OF INTRAVENOUS FLUIDS

4.1 INTRODUCTION

Acute protein-calorie malnutrition adversely affects the immune system and reduces the host's ability to launch an effective immune response against infectious agents (Section 1.6.1). Correction of nutritional deficiences by enteral feeding has been shown to improve immune competence in humans and experimental animals (Section 1.7.1). Also, there is evidence that parenteral nutrition with fat-free intravenous solutions can improve depressed immune parameters in malnourished humans and that such improvements are associated with significant clinical benefits (Section 1.7.2).

Although the effects on the immune system of malnutrition and subsequent nutritional repletion by total parenteral nutrition (TPN) have been studied extensively, the effects of the nutrient solutions themselves are largely unknown. The aims of this study were, firstly, to examine the effects of five commonly used intravenous nutrient solutions on the *in vitro* reactivity of normal lymphocytes, and secondly, to measure the reactivity of lymphocytes in patients receiving TPN.

4.2 LABORATORY STUDY

4.2.1 METHOD

Five parenteral nutrition solutions were examined (Table 4.1). Two composite solutions ("A" and "B") were made up so that they would be suitable for use in a clinical situation; they were almost isocaloric and contained similar amounts of nitrogen and electrolytes per litre, but differed in that solution "B" contained a fat emulsion ("Intralipid", Kabivitrum Ltd., Stockholm, Sweden) which provided approximately half of the calorie content. The other solutions contained the same amount of nitrogen (solution "C"), dextrose (solution "D"), and fat emulsion

Table 4.1 Composition of five test intravenous solutions (contents per litre)

SOLUTION	N (g)	Dextrose (g)	Fat (g)	Na (mmol)	K (mmol)	Cl (mmol)	Kilo-calories
A	4.7	200	0	24.3	20.0	23.3	922
B	4.7	89	44.3	23.0	25.0	21.6	868
C	4.7	0	0	24.3	20.0	23.3	366
D	0	200	0	0	6.0	0	800
E	0	0	44.3	0	0	0	400

(solution "E") as the composite solutions, and thereby acted as control solutions for either "A" or "B". Lymphocytes were obtained from five healthy adult volunteers (Section 2.3.3 (a)). Serial dilutions (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128) of the five test solutions were made by addition of Hank's BSS and each dilution was divided equally into five aliquots which were incubated separately with 0.5×10^6 lymphocytes (Figure 4.1). After incubation for 30 minutes at 23°C, lymphocytes were washed three times to remove all traces of the intravenous solutions, and lymphocyte reactivity to PPD was measured using the TEEM test (Section 2.3). The Student's t test was used to assess the significance of differences between results of in vitro lymphocyte reactions following incubation with the five solutions.

4.2.2 RESULTS

The effect of each dilution of the five test solutions on in vitro reactivity of normal lymphocytes is shown in Figure 4.2. Solutions "A" (synthamin and dextrose), "C" (synthamin), and "D" (dextrose) caused inhibition of lymphocyte reactivity at high concentrations only, and at a 1 in 4 dilution these three solutions had no effect on lymphocyte responsiveness. In contrast, solutions "B" and "E", which contained a fat emulsion, initially produced a significant increase in lymphocyte reactivity (Student's t test, $p<0.001$). Highly significant inhibition occurred with 1/4, 1/8, 1/16 and 1/32 dilutions ($p<0.001$) and the depressive effect of these fat-containing solutions diminished with further dilution.

Maximal inhibition of lymphocyte reactivity occurred when the triglyceride content of the test solution was 0.28 - 1.13 mmol/l (1/4 - 1/16 dilutions). The recommended infusion rate of "Intralipid" results in

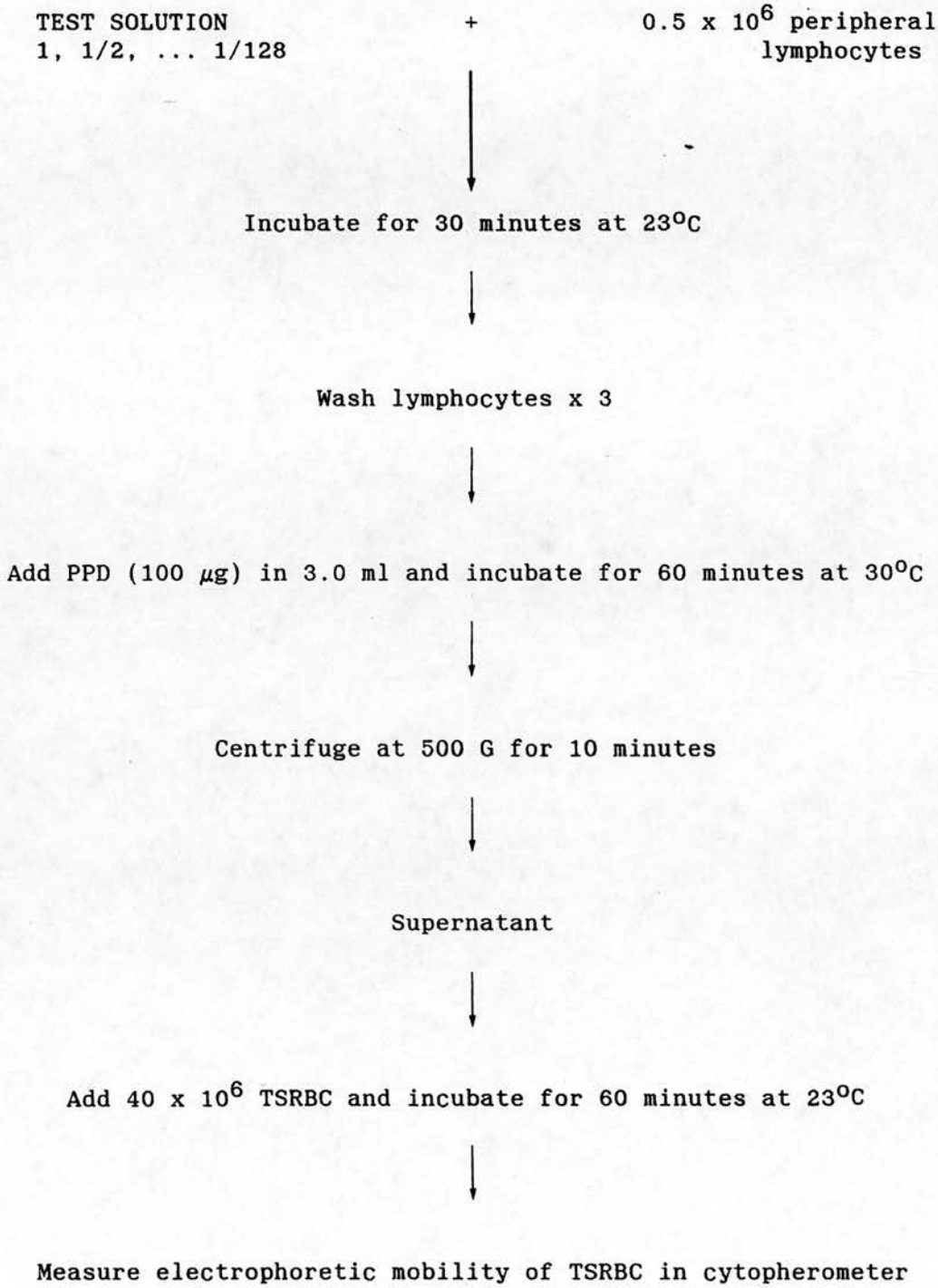


Figure 4.1 Method of measuring the effect of intravenous nutrient solutions on <u>in vitro</u> lymphocyte reactivity

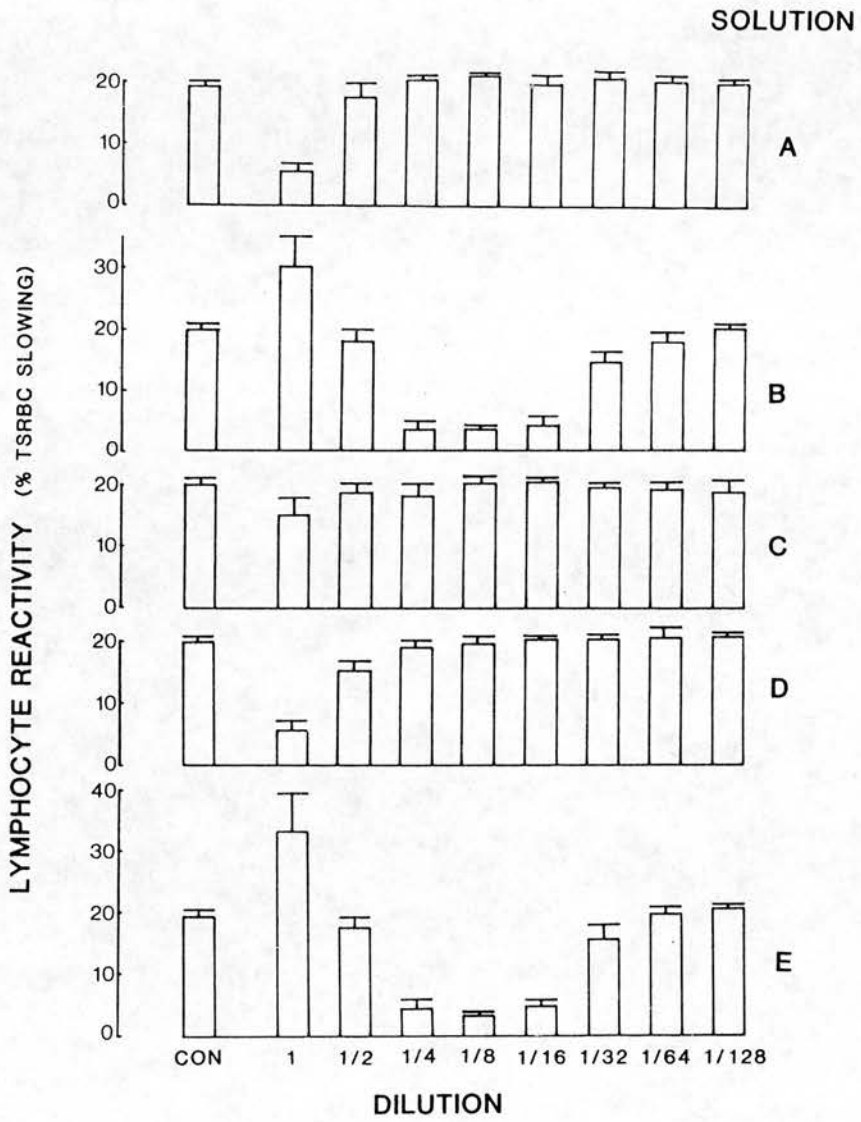


Figure 4.2 The effect of five intravenous nutrient solutions on <u>in vitro</u> lymphocyte reactivity to PPD (mean ± 1SD)

Solution A = Synthamin and Dextrose
Solution B = Synthamin, Dextrose and Intralipid
Solution C = Synthamin Solution
Solution D = Dextrose
Solution E = Intralipid
Con = Control

a plasma triglyceride range of 0.22 - 0.90 mmol/l[360], levels which coincide closely with the range of maximal inhibition of lymphocyte reactivity. MacFadyen et al[361] have shown that infusion of 500 ml Intralipid 10% over four hours into adult patients produces elevated plasma triglyceride concentrations with peak levels of around 0.44 mmol/l. Thus, the intravenous infusion of Intralipid for parenteral nutrition may produce plasma concentrations of triglycerides which correspond almost exactly to the levels that produce maximum inhibition of lymphocyte reactivity *in vitro*.

4.3 CLINICAL STUDY

A clinical study was undertaken to determine whether *in vitro* lymphocyte reactivity of patients receiving intravenous nutrient solutions was affected by the feeding solution.

4.3.1 METHOD

The effect of three intravenous fluid regimes on lymphocyte reactivity was studied in 15 female patients who had undergone elective cholecystectomy for radiologically-proven cholelithiasis (Table 4.2). As nutritional impairment adversely affects lymphocyte reactivity, only well nourished subjects suitable for a short period of parenteral nutrition were studied. Informed consent was obtained from each patient.

A central venous catheter was inserted via an antecubital fossa vein after induction of general anaesthesia. All patients underwent cholecystectomy without exploration of the common bile duct. A period of six hours was allowed after operation for haemodynamic stabilisation before the intravenous feeding regimes were commenced. No patient required a blood transfusion.

Table 4.2 Patient details

	GROUP 1	GROUP 2	GROUP 3
n	5	5	5
Age (years) mean ± 1SD	63.5 ±7.3	64.3 ±2.5	63.8 ±5.6
Weight (Kg) mean ± 1SD	62.5 ±9.4	59.8 ±5.4	64.9 ±7.5
PW/IBW (%) mean ± 1SD	119.6 ±17.6	115.8 ±13.0	122.8 ±15.2
TSF (%) mean ± 1SD	121.3 ±22.5	117.6 ±32.1	128.3 ±30.0
AMC (%) mean ± 1SD	91.6 ±8.2	90.5 ±4.5	99.1 ±10.5

Group 1 = Control
Group 2 = Dextrose as principal calorie source
Group 3 = Intralipid in addition to Dextrose

PW/IBW (%) = Patient's weight expressed as a percentage of ideal body weight for height.

TSF (%) = Triceps skin fold thickness as a percentage of standard (Metropolitan Life Tables)

AMC (%) = Arm muscle circumference as a percentage of standard

Patients were allocated randomly to one of three groups (Table 4.3). Group 1 received a standard daily maintenance intravenous fluid regime of 1 litre of 0.9% saline and 2 litres of 5% dextrose for two days post-operatively, group 2 received a TPN regime of amino acids and dextrose, and group 3 received a TPN regime which included a 20% fat emulsion. The amino acid and dextrose solutions were prepared in three litre polyvinylchloride bags by the hospital pharmacy and were infused through central venous catheters at a rate controlled by volumetric infusion pumps. The fat emulsion was given through a 'Y' connector at a point just before the central venous catheter penetrated the skin. Infusions were continued until 48 hours from the commencement of surgery, during which time patients were allowed to take water by mouth as desired.

Venous blood samples were taken from each patient immediately before operation (day 0) and on the first, second, third and seventh post-operative days. Each sample was divided into two aliquots, one to provide lymphocytes and the other to provide serum for measurement of triglycerides and free fatty acids. Lymphocyte reactivity to PPD was measured using the TEEM test (Section 2.3.3). The Wilcoxon Rank sum test was used for statistical analysis.

4.3.2 RESULTS

Lymphocyte responses to PPD of the three groups did not differ significantly before operation (Figure 4.3). The responses of all patients fell on the first and second post-operative days. Those patients receiving amino acids, dextrose and fat (group 3) had significantly lower lymphocyte responses than those receiving physiological saline (group 1) during the period of infusion ($p<0.05$). Although the lymphocyte responses of group 3 were lower than those of group 2 on the first and second post-operative

Table 4.3 Composition of post-operative intravenous fluid regimes

	GROUP 1 ("Control")	GROUP 2 ("Dextrose")	GROUP 3 ("Fat")
Synthamin 14	0	1000 ml	1000 ml
Dextrose 5%	2000 ml	0	0
Dextrose 20%	0	500 ml	1500 ml
Dextrose 50%	0	1000 ml	1000 ml
Intralipid 20%	0	0	667 ml
Saline 0.45%	0	500 ml	0
Saline 0.9%	1000 ml	0	0
Contents per 24 hours:			
Volume (ml)	3000	3000	3167
Nitrogen (g)	0	14.3	14.3
Kilocalories	400	2766	2766
Sodium (mmol)	150	111	73
Potassium (mmol)	0	60	60
Chloride (mmol)	150	108	70

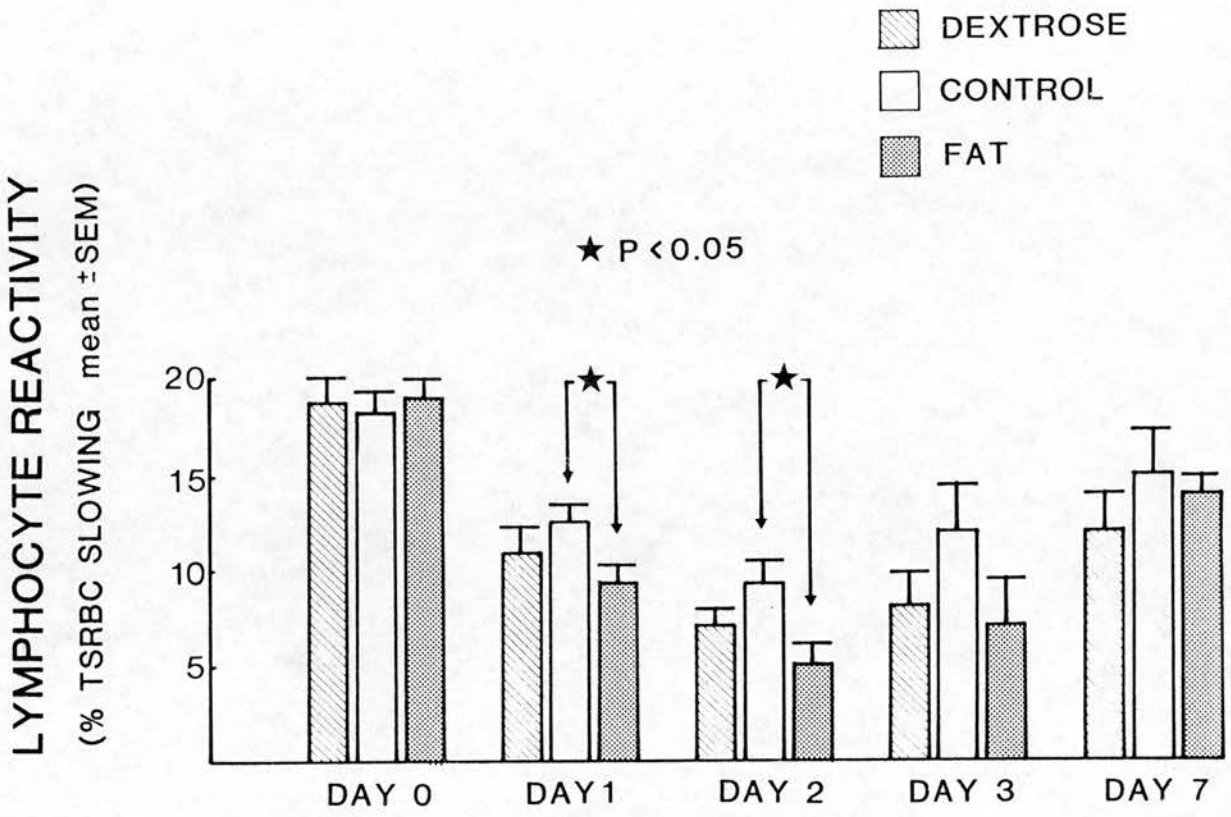


Figure 4.3 The results of <u>in vitro</u> lymphocyte responses to PPD in patients receiving either normal saline and dextrose (control) or parenteral nutrition with either dextrose or fat emulsion.

days, the differences failed to reach statistical significance at the 5% level. On the third post-operative day, after cessation of all intravenous regimes, lymphocyte responses were slightly higher overall than on the previous day; responses of group 3 were lower than those of groups 1 and 2, although the differences were not significant. By the seventh day lymphocyte responses had partly recovered and were approaching pre-operative levels.

The serum triglyceride and free fatty acid concentrations are shown in Tables 4.4 and 4.5 respectively. There was no statistically significant difference between the serum triglyceride concentrations of the three groups for any of the days of the study. The serum free fatty acid (FFA) concentrations in patients receiving the fat emulsion (group 3) increased significantly during the period of infusion ($p<0.001$). A similar rise was not observed in patients who received fat-free solutions (groups 1 and 2).

4.4 DISCUSSION

The study has shown that *in vitro* lymphocyte reactivity is impaired, firstly, after short-term incubation with "therapeutic" concentrations of a fat emulsion, and secondly, in patients who are receiving a fat emulsion by infusion as part of a short TPN regime. The results are in keeping with the known effects of fatty acids on *in vitro* and *in vivo* tests of lymphocyte function.

The mechanism of the inhibitory effect of PUFA is not known. As some fatty acids are precursors of prostaglandins, their inhibitory activity may be linked closely with that of prostaglandins. If so, PUFA might exert their influence at cell surface prostaglandin receptors and activate membrane-bound adenyl cyclase to increase intracellular levels of adenosine 3'5' cyclic monophosphate (cyclic AMP), which in turn would

Table 4.4 Serum triglyceride concentration of patients receiving post-operative intravenous fluids

GROUP	PATIENT	SERUM TRIGLYCERIDE CONCENTRATION (mmol/l)				
		Day				
		0	1	2	3	7
1	1	0.20	1.12	1.47	1.91	1.67
(Control)	2	2.39	0.79	1.65	1.71	2.38
	3	2.69	1.20	1.08	1.00	1.36
	4	2.16	0.39	0.24	1.18	1.23
	5	1.52	1.30	1.12	0.97	1.41
	mean	1.79	0.96	1.11	1.35	1.61
	1SD	0.88	0.33	0.48	0.38	0.41
2	1	1.35	0.18	1.06	0.18	1.98
(Dextrose)	2	1.36	0.61	0.98	1.31	0.53
	3	1.71	0.86	1.50	2.12	2.77
	4	1.74	0.68	0.66	1.11	1.40
	5	0.89	0.76	1.29	0.93	1.30
	mean	1.41	0.62	1.09	1.13	1.59
	1SD	0.30	0.23	0.28	0.62	0.74
3	1	1.41	1.46	1.96	1.03	0.98
(Intralipid)	2	0.96	1.48	1.46	1.98	0.97
	3	1.71	0.85	1.51	2.12	2.77
	4	1.74	0.68	0.66	0.40	1.41
	5	0.89	0.76	1.37	1.00	1.36
	mean	1.34	1.05	1.19	1.30	1.49
	1SD	0.36	0.35	0.58	0.65	0.66

Reference range: 0.1 - 1.8 mmol/litre.

Table 4.5 Serum free fatty acid concentrations (meq/litre) of patients receiving post-operative intravenous fluids

GROUP	PATIENT	SERUM FREE FATTY ACID CONCENTRATIONS (meq/l)				
		Day				
		0	1	2	3	7
1	1	1.45	0.46	0.54	1.08	0.37
(Control)	2	0.72	0.83	0.54	0.92	0.29
	3	0.76	2.28	1.40	1.22	0.51
	4	0.50	0.27	1.02	0.75	0.83
	5	0.90	0.71	0.67	1.33	0.78
	mean	0.86	0.91	0.83	1.06	0.56
	1SD	0.31	0.71	0.33	0.20	0.22
2	1	0.41	1.91	2.02	2.01	0.39
(Dextrose)	2	0.71	1.38	0.15	1.53	1.50
	3	0.77	1.16	1.11	0.81	1.82
	4	0.69	0.65	0.40	1.47	0.66
	5	0.66	0.54	0.89	0.32	0.59
	mean	0.64	1.13	0.91	1.22	0.99
	1SD	0.12	0.50	0.65	0.59	0.56
3	1	1.29	1.40	1.22	1.40	0.67
(Intralipid)	2	0.76	1.64	1.63	1.35	0.66
	3	0.83	1.81	2.29	0.21	0.48
	4	0.46	1.90	2.10	1.48	0.72
	5	0.83	1.00	1.06	1.29	1.05
	mean	0.83	1.55*	1.66*	1.14	0.72
	1SD	0.26	0.32	0.48	0.47	0.19

Reference range: 0.09 - 0.8 meq/litre.

* Day 0 x Day 1, and Day 0 x Day 2: $p<0.05$ Wilcoxon Rank Sum Test

activate an intracellular messenger system. Alternatively, PUFA could be converted rapidly to prostaglandin at the lymphocyte surface by the membrane-situated prostaglandin synthetase enzyme complex. The study indicates that suppression can be effected by contact between lymphocytes and PUFA for a short period of time and so this would tend to favour the first proposed mechanism of inhibition.

Intralipid significantly inhibited in vitro lymphocyte reactivity to PPD antigen, a result which is in keeping with that of Mertin et al[300,301]. It is possible that the fatty acids were bound to the lymphocyte cell surface and non-specifically blocked receptor sites for PPD. Although lymphocytes were washed three times after incubation with Intralipid, it is possible that PUFA remained on the lymphocyte surface in spite of washing.

There have been few studies of the in vitro effects of solutions used for parenteral nutrition. Ota et al[362] studied the effects of 10% Intralipid on lymphocyte transformation, as measured by incorporation of H^3-thymidine following in vitro stimulation with PHA and Varidase. They found that at a concentration of 1.15 mg Intralipid per ml (equivalent to 0.6 mg of esterified linoleic acid per ml) Intralipid significantly increased lymphocyte reactivity, although two other concentrations (0.15 mg/ml and 2.3 mg/ml) did not affect lymphocyte function. Ota[362] also studied the effect of free linoleic acid on lymphocyte transformation and found that it caused a 75% decrease in transformation, confirming the findings of Mertin[300]. The experimental system used by Ota differed markedly from that of the present study. Also, in Ota's transformation studies, lymphocytes were incubated for three days (PHA) and five days (Varidase) in the presence of antibiotics and human serum, in addition to the Intralipid solutions. Closer examination of Ota's results reveals that

in five of the six experiments some lymphocyte cultures were inhibited by Intralipid[362]. Ota did not attempt to explain his results which are in disagreement with the known immunosuppressive effects of some of the components of Intralipid.

The clinical study demonstrated that well nourished females undergoing elective surgery of moderate severity and receiving post-operatively a fat emulsion as a source of calories had significantly lower lymphocyte reactivity during the period of infusion than two matched groups of controls. The numbers in each group were small but nevertheless the differences in lymphocyte reactivity between the groups receiving and not receiving Intralipid were statistically significant. Although the study does not allow any conclusions to be drawn regarding possible clinical sequelae, the demonstration that fat emulsion has non-specific immunosuppressive properties may have implications for patients who are candidates for TPN and who are likely to have impaired cellular immunity before starting therapy. While evidence suggests that restoration of nutritional status by prolonged TPN with fat-free solutions can improve immune function, the short-term effects of intravenous fat emulsions appear to be detrimental to host immunity.

Sepsis has been reported after prolonged treatment with Intralipid[363] which has also been shown to adversely affect other immunological mechanisms, such as the bactericidal capacity of granulocytes[364]. Thus, extensive use of fat emulsions with a high content of fatty acids could result in further impairment of immune function in already immunocompromised patients and expose them to even greater risks of septic complications. Further studies are required to determine the clinical significance of these findings.

Serum triglyceride levels did not rise in subjects receiving an intravenous infusion rich in triglycerides because the rate of Intralipid infusion was less than the potential clearance rate of triglyceride from the circulation. The infusion rate of triglyceride in the patients receiving Intralipid was 92.6 μmol triglyceride/minute, which would lead to an increase in plasma triglyceride concentration of 33.0 μmol triglyceride/litre/minute (Appendix 15). This increase is below the rate of clearance of exogenous lipids from the circulation: in man, exogenous fats are removed at rates which depend on the concentration of the fat in plasma: above 1.0 mmol/l the rate of clearance of triglyceride is 0.07 mmol/l of plasma/minute, and below this level clearance is slightly lower at 0.06 mmol/minute[365]. Thus, the rate of infusion of triglyceride was well within its rate of clearance. MacFadyen et al[361] demonstrated that triglycerides do accumulate in plasma when their rate of infusion exceeds their rate of clearance.

Serum FFA concentration rose significantly during infusion of Intralipid. The reason for this rise is complex and is not due simply to excessive infusion of FFA, which constitute only 1% of Intralipid. The particles of an infused fat emulsion are phagocytosed by cells of the reticulo-endothelial system and in this way are cleared from the blood stream[366]. The intracellular enzyme lipoprotein lipase hydrolyses triglyceride to FFA and glycerol within the phagocytes. The turnover of FFA in plasma is rapid[367]. FFA are metabolised in the liver to form ketone bodies and in adipose tissue and peripheral tissues where they form acetyl CoA which is used in the re-synthesis of fatty acids. Thus, FFA in plasma following intravenous infusion of fat may be derived from either triglycerides or infused FFA. In this study FFA were infused at the rate of 2.82 meq FFA per day. Assuming a plasma volume of 2.79 l (calculated

from mean body weight), this infusion would cause a rise in plasma FFA concentrations after one day of 1.01 meq/l if there was no FFA clearance during that time. In addition, the FFA potentially available from hydrolysis of triglycerides is nearly 100 times the infused quantity. However, the mean measured increase in plasma FFA after the first 24 hours of fat infusion was only 0.72 meq/l, indicating that removal of FFA is an extremely rapid and efficient process.

4.5 SUMMARY

Although nutritional repletion of malnourished individuals may improve tests of cell-mediated immunity, the effects of intravenous nutrient solutions themselves on the immune system are largely unknown. The effects of five parenteral nutrition solutions on *in vitro* lymphocyte reactivity were measured by incubating normal human lymphocytes with dilutions of (a) an amino acid/dextrose solution, (b) an amino acid/dextrose/fat solution, (c) an amino acid solution, (d) dextrose, and (e) a fat emulsion, and lymphocyte responses to PPD were measured using the TEEM test. Lymphocytes were inhibited significantly by the fat emulsion at concentrations identical to those achieved by therapeutic infusion. Lymphocyte reactivity was measured in 15 post-operative patients allocated randomly to receive either simple electrolyte solutions or iso-caloric parenteral regimes with or without a fat emulsion. *In vitro* lymphocyte reactivity was significantly depressed in patients during infusion of fat emulsion in comparison with controls ($p<0.05$). The results show that fat emulsion impairs lymphocyte reactivity and suggest that careful consideration should be given before using fat emulsions in patients in whom cell-mediated immunity is impaired already.

CHAPTER 5

PLASMA SUPPRESSIVE ACTIVITY AND TUMOUR GROWTH

5.1 INTRODUCTION

The studies reported in Chapter 3 showed that lymphocyte SA in plasma was increased significantly in patients with malignant disease, and that the increased PSA was due mainly to an alteration in the biological behaviour of A2M as well as IC and small MW substances. It is likely that these lymphocyte suppressive factors are directly or indirectly related to the presence of malignant tissue. The role of these suppressive substances is speculative but it is proposed by the author that they represent mechanisms of "immunological escape" and a means by which tumour growth is enhanced (Section 1.8).

If this hypothesis is correct, then it should be possible to demonstrate a causal relationship between increased PSA and the presence of a tumour. The ideal confirmatory study would measure PSA sequentially in a large number of subjects over a long period of time and compare values in those who did and did not develop cancer. Clearly, such a study was not feasible, and so the author turned to an animal model. Secondly, if a high PSA is beneficial for tumours, manipulation of PSA might be expected to influence the growth of tumours. This hypothesis was examined in an animal model also.

The present chapter describes two animal experiments which examined (a) the causal relationship between tumour growth and PSA, and (b) the effect of increased PSA on tumour growth.

5.2 METHODS

5.2.1 EXPERIMENTAL ANIMALS

Rats were used as experimental animals because they were easy to maintain and use, they could support the growth of a readily available tumour (the growth pattern of which was known), they could be bled

repeatedly without undue stress, and their MHC types were known. Two inbred rat strains were used: (i) Wistar-Albino-Boots (WAB) rats were used as recipients of blood transfusions and tumours, and (ii) Portland-Virol-Glaxo (PVG/C) rats were used as allogeneic blood donors for WAB recipients. MHC designations are shown in Table 5.1.

All rats were adult females and weighed between 200 - 250 grams. PVG/C rats tended to be slightly heavier than the WAB rats. PVG/C rats were white with black 'hoods', while WAB rats were completely white. Animals were housed four to a cage in an air-conditioned room at 24^{o}C, with a 12 hour light - 12 hour dark cycle, for one week before and during the experiments.

5.2.2 TUMOUR

A rat sarcoma was used (gift of Dr. Allan Simpson, Department of Clinical Biochemistry, Royal Victoria Infirmary, Newcastle Upon Tyne, England). The sarcoma had been induced in an adult female WAB rat by subcutaneous injection of 3-methylcholanthrene (5 mg) in trioctanoin and maintained by subpassage into syngeneic rats of the same sex every two weeks. The tumour grew rapidly and did not metastasise within the first three weeks after passage and was highly immunogenic. Baldwin et al[368] found that WAB rats immunised with the sarcoma, which was subsequently excised, were able to reject a later challenge with 5 x 10^6 tumour cells or whole tumour grafts.

A tumour suspension was made by excising a single sarcoma, removing the surrounding connective tissue and finely mincing the tumour; the homogenate was diluted with Hank's BSS containing penicillin 200 units/ml so that the final concentration was 1 x 10^7 cells/ml. Cell viability was

Table 5.1 MHC designations of experimental animals

RAT	Ag B	H-1	MLR	Ag C
WAB	2	W	2	2
PVG/C	5	C	5	2

Ag B and H-1 : serologically defined antigens

MLR : Antigens defined by mixed lymphocyte reaction

Ag C : red blood cell antigen

checked by trypan blue exclusion and 90% of cells in the suspension excluded the dye. The tumour suspension was placed into 1.0 ml sterile syringes marked with 0.01 ml gradations. Each animal was injected with 0.1 ml of tumour suspension (1×10^6 cells) into the subcutaneous tissue of the left flank.

Tumours were palpable as small discrete nodules by day six, and grew as spherical or ovoid lumps in the subcutaneous tissue of the flank, ideally situated for easy measurement. Tumours were not allowed to grow beyond 3 x 3 cm, a size which could be attained usually within 21 days of passage. During this time metastases were not seen. Once a tumour approximated to 3 x 3 cm, it was either excised, or the animal was sacrificed by barbiturate overdose and a thorough necropsy performed to search for metastases.

5.2.3 ANAESTHETIC

Venepuncture, infusion of saline, blood transfusion, and tumour passage were performed under general anaesthesia. Animals were placed individually in a large glass flask which was then covered. The base of the flask contained a thick pad of cotton wool soaked with ether and rats became unconscious after 20 - 30 seconds. Anaesthetised animals were removed from the flask and placed supine on a cork board with each limb strapped to the board with 1/2 inch adhesive tape. Anaesthesia was maintained by administering ether on a cotton wool pad at the apex of a small cone which was placed over the nose. Rats were conscious within a few minutes of removing the anaesthetic nose cone and were able to walk around in their cages within 15 - 20 minutes of finishing the anaesthetic. The rats withstood anaesthesia well and no deaths were attributed to the anaesthetic.

5.2.4 VENEPUNCTURE

Animals were bled from the jugular vein under general anaesthesia. Each rat was placed supine on a cork board and a 1 cm transverse incision was made on the upper part of the chest on one or other side to expose the pectoralis muscle. The jugular vein was located beneath the fine layer of fascia at the upper border of the pectoralis muscle. A sterile 2.0 ml or 5.0 ml plastic syringe was filled with a solution of heparin (1000 units in 1 ml) and then emptied so that there was no heparin within the barrell of the syringe but some heparin remained in the part which locked into the hub of the attached 21 gauge needle. The needle was passed through the uppermost part of the muscle into the jugular vein. In this way, the upper edge of the muscle covered the point of puncture in the vein and prevented bleeding after removal of the needle.

Samples of blood (5.0 - 7.0 ml) taken from PVG/C and WAB donor animals were used immediately for transfusion into recipient animals. Following removal of more than 4 ml of blood an equal volume of 0.9% saline was infused into the anaesthetised animal. WAB donor rats were not used later as recipient animals. Blood samples were taken from WAB recipients for measurement of lymphocyte reactivity and plasma SA. Aliquots of venous blood (0.3 ml and 0.7 ml) were placed separately in plastic blood storage tubes containing lithium heparin before assay by the TEEM test.

After exposure of the vein and venepuncture, each skin wound was closed with two interrupted black silk sutures. The wounds healed well and repeated venepunctures could be performed at the same site on each side of the neck without adverse effects on the animals.

5.2.5 TUMOUR MEASUREMENT

After passage of tumour cells, rats were inspected daily and the

left flank palpated to detect the presence of a tumour nodule. A small discrete nodule was present in all animals by the sixth day and was large enough to measure. Measurements were performed daily from the sixth day until the tumours were excised.

The aim of sequential measuring was to assess the increase in tumour mass. However, the mass of a growing tumour cannot be measured directly in vivo. The net weight of a tumour-bearing animal can be measured but any change in the animal's weight may reflect factors other than tumour mass. The growth of an animal tumour is often expressed in terms of increases in measurable diameters, but although the horizontal and vertical diameters of a tumour may be measured accurately, the depth or thickness of a tumour is difficult to measure accurately in situ. Because of the inaccuracies inherent in measuring three axes of in situ spherical or ovoid tumours, a method was used whereby tumour volume was calculated from measurement of two axes of the tumour.

Assuming a fairly constant relationship of tumour mass with tumour volume, the growth of a tumour can be expressed in terms of increases in tumour volume. The expression of growth by changes in tumour dimensions, rather than tumour volume, cannot accurately describe increases in tumour mass, because increases in the dimensions of an object cause a greater proportional increase in volume as the dimensions become larger.

The formula used to estimate tumour volume was based on work performed by Perri et al[369] who found good correlation between calculated volumes and weights of ovoid-shaped sarcomas in rats using the formula:

$$V = a\,b^2$$

where V = calculated volume of tumour

a = the length of the major axis, and

b = the height and width of the minor axes

However, this formula assumes that the height and width of the tumour (the minor axes) are equal. Attia et al[370] investigated the application of this formula to the measurement 122 murine spontaneous mammary tumours: they measured tumour volume by liquid displacement and correlated the results with values obtained from the formula of Perri ($V = ab^2$), calculated from measurements of the tumours in situ immediately before excision. They found a strong correlation between the measured and calculated volumes. Their observed regression co-efficient of the regression equation describing the relationship between measured and observed volumes was 0.3986. This figure was converted to 0.4 for practical purposes, and the regression equation formulated by Attia et al[370] was:

$$y = n + mx$$

$$y = 0 + 0.4x$$

or $$y = (0.4)\ (ab^2)$$

where y = measured tumour volume,

n = distance above the abscissa at which the regression line intercepts the vertical axis. (The value of n was zero as the regression line passed through the point of interception of the x and y axes).

m = regression co-efficient, signifying the amount by which a change in x must be multiplied to give the corresponding change in y, (in this case m = 0.4).

x = tumour volume calculated from the formula $V = ab^2$.

Attia et al[370] were able to show that the measured tumour volumes were very close to tumour volumes (V) calculated from the equation $V = (0.4)\ (ab^2)$.

It is apparent, therefore, that the depth dimension of an ovoid tumour bears a constant relationship to the value of the minor axis (b), although it may not equal the value of b. The formula of Attia was used to calculate tumour volumes. Each tumour was measured daily from the sixth day using calipers. The skin thickness of the right flank was measured daily also, and subtracted from measured diameters of the tumour on the left flank.

5.3 SEQUENTIAL MEASUREMENT OF PLASMA SUPPRESSIVE ACTIVITY IN TUMOUR-BEARING RATS

An experiment was performed to examine the relationship between plasma suppressive activity (PSA) and tumour growth. It has been demonstrated that a high PSA is present in patients with cancer but the precise relationship of PSA with tumour growth has not been defined. This experiment measured PSA in rats before, during and after the growth of a syngeneic tumour.

5.3.1 METHOD

Twelve WAB rats were studied. Each rat was injected subcutaneously with 1×10^6 viable cells from a single methyl cholanthrene-induced sarcoma (Section 5.2.2) on day 1. Tumours were measured daily and were excised completely under general anaesthetic on day 14. Tumours were cleaned of surrounding connective tissue and weighed. All animals were allowed to recover from anaesthesia after the flank wounds had been sutured. Venepunctures were performed on each animal on days 1 (immediately before tumour transplantation), 10, 14 (immediately before excision of tumours), 17, 24, 32, 40, 50, 60, 80 and 130. On each occasion 1.0 ml of blood was obtained and divided into two aliquots, 0.3 ml and 0.7 ml, which were used

to measure PSA and lymphocyte reactivity to PPD respectively with the TEEM test.

5.3.2 RESULTS

The PSA values before tumour inoculation, during tumour growth and after tumour excision are presented in Table 5.2. The results have been plotted graphically in Figure 5.1 as the log of the microlitre volume of plasma, and inverted on the vertical axis so that a rise in PSA is shown as a rise in the points plotted on the graph.

PSA was low before tumour inoculation (day 1) but increased dramatically during tumour growth (days 10 and 14). Tumours were excised after venepuncture on day 14 and PSA decreased subsequently to reach pre-tumour levels by day 32. PSA remained at the relatively low pre-tumour level thereafter. Local recurrence did not occur in any animal. However, one rat was observed on day 76 to have a mass at the angle between the left fore-limb and the chest wall. A blood sample was taken from the animal for lymphocyte reactivity and PSA estimation, and the rat was sacrificed. Necropsy revealed that the mass was a tumour nodule and that both lungs contained multiple metastatic deposits. The PSA of this rat was increased significantly at 2.2 μl plasma, although the PSA had returned to pre-tumour levels after excision of its flank tumour.

Lymphocyte reactivity was initially high in all animals (Figure 5.2) but fell markedly after tumour inoculation. Lymphocyte reactivity recovered slowly following complete excision of the tumours and returned to pre-tumour levels by day 40, a little later than PSA. Unfortunately, lymphocytes from the rat which developed lymph node and pulmonary metastases were lost during preparation before their reactivity

Table 5.2 Plasma suppressive activity (PSA) before (day 1), during (days 10 and 14) and after (days 17 - 130) tumour growth

DAY	n	PSA (µl plasma) mean ± 1SD
1	12	35.9 ± 1.61
10	12	1.08 ± 0.05
14	12	0.59 ± 0.06
17	12	1.56 ± 0.42
24	12	2.32 ± 0.12
32	12	36.1 ± 0.52
40	12	35.3 ± 0.12
50	11	35.2 ± 0.45
60	11	36.6 ± 1.40
80	10	35.7 ± 0.35
130	10	35.1 ± 0.09

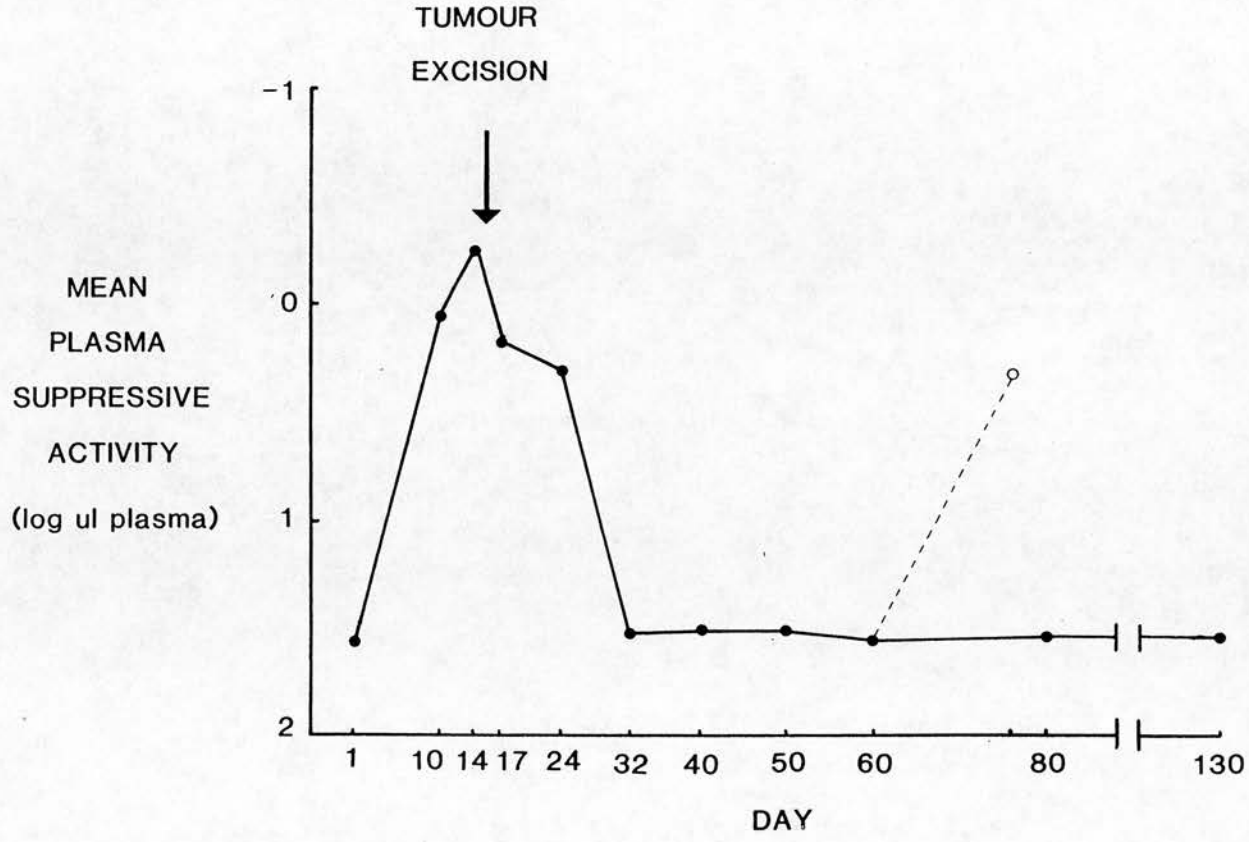


Figure 5.1 Mean plasma suppressive activity (Log μl plasma) in 12 WAB rats before, during and after tumour growth.

Open circle represents the rat which developed metastases.

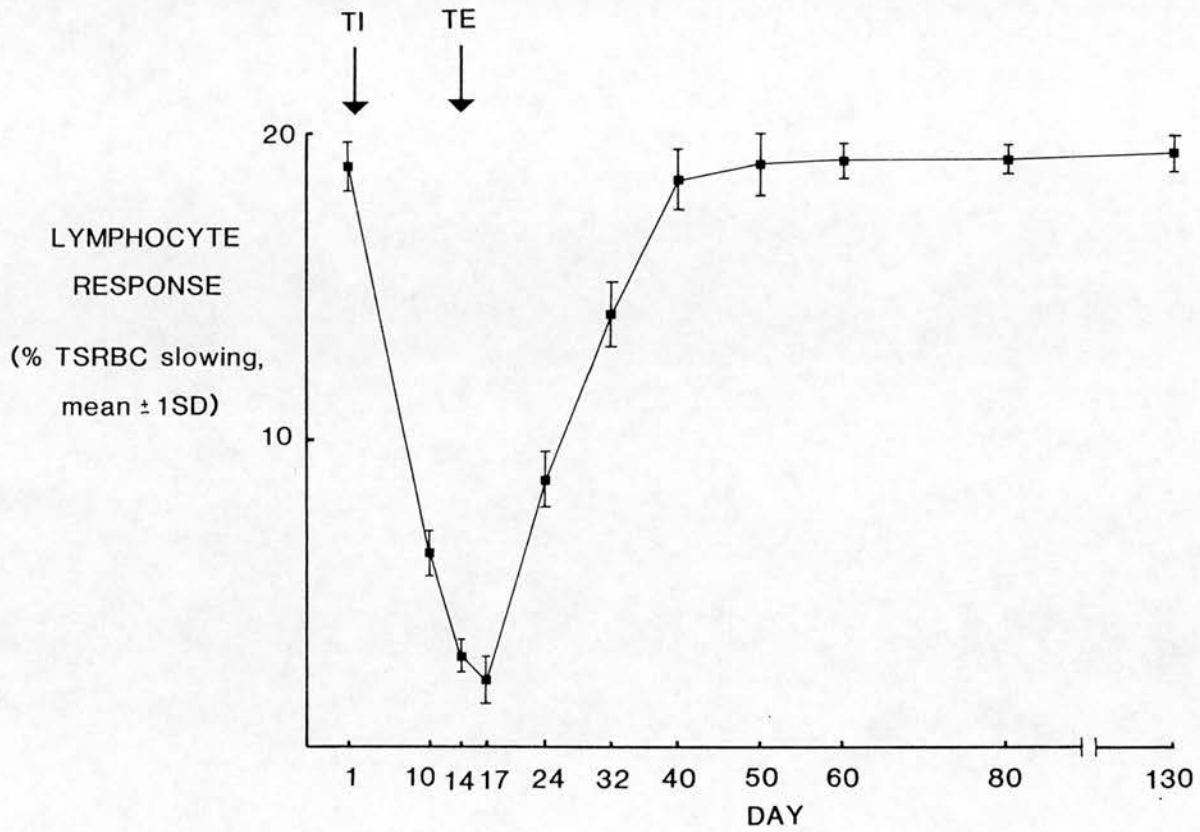


Figure 5.2 Lymphocyte responses (mean ± 1SD) to PPD in 12 WAB rats before, during and after tumour growth

TI = Tumour inoculation
TE = Tumour excision

was measured.

5.3.3 DISCUSSION

This experiment demonstrated that immunosuppressive activity can be detected in plasma of tumour-bearing WAB rats and that the rise and fall in PSA accurately paralleled tumour growth in all animals. All animals, except the one with regional lymph node metastases, remained well and tumour-free for seven months after excision, suggesting that these rats were "cured" of their tumours. The case of metastatic spread is the first to be observed with this tumour in our laboratory. PSA was grossly elevated at the time of detection of metastases, indicating that a second increase in PSA was a reliable index of recurrent disease in this animal. Unfortunately, PSA was not measured frequently enough to detect an increase in PSA before metastases became obvious.

The fall in PSA after removal of the primary tumours was relatively slow. The significance of this is uncertain but is may reflect the elimination of naturally occurring or tumour-induced immunosuppressive substances from the host.

Although the precise significance of PSA is uncertain, it has been demonstrated that plasma from a tumour-bearing host is able to suppress the reactivity of lymphocytes from non-tumourous syngeneic animals. This indicates that a high PSA associated with a growing tumour contributes to host immunosuppression. Other cellular immune parameters may be affected in addition to lymphocyte function. Immune suppression in some animal tumour models results in increased tumour growth, and it is likely that a high level of PSA inhibits or suppresses host immune defence mechanisms, and thereby accelerates tumour growth.

Lymphocyte responses were impaired progressively as tumours

increased in size. Impairment could have been due to circulating immunosuppressive factors which caused the increase in PSA or to an intrinsic defect of the lymphocytes. However, the reduction in lymphocyte reactivity occurred within a few days of tumour inoculation, within the life span of a lymphocyte; this would favour an 'extrinsic' cause for the lymphocyte impairment.

The association of raised PSA with increasing tumour growth was observed in all animals. The only animal to develop metastases was found to have a greatly elevated PSA when the metastases were observed. No conclusions can be drawn from a single animal, but the observation suggests a direction for future experiments involving sequential measurements of PSA in an animal model which more closely resembles the human situation, i.e. using a metastasising tumour.

5.4 MANIPULATION OF PLASMA SUPPRESSIVE ACTIVITY

The previous experiment demonstrated that PSA paralleled tumour growth, although the significance of increased PSA in terms of benefit to the tumour is not known with certainty. It can be postulated that the increase in PSA, which is found in humans and experimental animals with tumours, benefits tumour growth by way of its suppressive effect on the host's immune system. If this hypothesis is correct, then the increase in PSA associated with tumours may be viewed as a mechanism by which tumours escape from immune destruction. An experiment was performed to assess the influence of increased PSA on tumour growth.

The aim was to increase PSA artificially and, with appropriate controls, observe the effect on tumour growth. A method had to be found whereby PSA could be increased without altering other physiological

parameters. There are several possible methods. The immunosuppressive properties of naturally occurring proteins (including the alpha-globulins) have been reported in pregnancy[371], uraemia[344], recipients of renal allografts[372], diabetes[373] and haemophiliacs who have received blood transfusions [374]. However, there is some physiological or pathological event taking place in each of these examples to cause the increase in immunosuppressive activity. It would not have been possible to use a disease process to induce PSA in an animal model because the disease process could have altered immunological parameters apart from any effect on PSA, and also it would have been impossible to regulate a disease process so that similar levels of PSA were achieved in all experimental animals. Similarly, a tissue injury could have induced a raised PSA as a result of increased acute phase reactant proteins, but this method would have been open to the additional criticism that superadded infection (which would have been difficult to prevent in an area of tissue necrosis in an experimental animal) could have interfered with the experimental results. The use of chemicals or drugs to induce a high PSA was considered but was thought to be unsuitable as these agents themselves might have a direct effect on tumour growth.

It was extremely difficult to conceive a means of inducing a high PSA in an animal while at the same time maintaining its health and nutritional status. Animal studies[374] performed previously in our laboratory have shown that transfusion of allogeneic blood results in significant increases in PSA (the technique had been used extensively in the investigation of rejection of cardiac allografts in rats). The method has distinct advantages over the previously described methods, as well as some disadvantages. These are discussed later. The limitations of the blood transfusion technique were realised and accepted so that the study

could be performed.

5.4.1 METHOD

There were three experimental groups with 12 WAB rats in each:

(1) group 1 ("saline") received intravenous infusions of 0.9% saline.

(2) group 2 ("syngeneic") received intravenously whole blood from healthy, non-tumour bearing, syngeneic WAB rats.

(3) group 3 ("allogeneic") received intravenously whole blood from healthy, non-tumour bearing, PVG/C donor rats.

Each infusion (saline) or transfusion (syngeneic or allogeneic) was 2.0 ml in volume. Each recipient animal was given two infusions or transfusions separated by an interval of three days. Recipient WAB rats were bled on days 1, 4, 18, 28, 32, 35, 42, 50, 57, 67, 80 and 100. On each occasion a 1.0 ml blood sample was obtained for measurement of lymphocyte reactivity and PSA. Immediately after venepuncture on days 1 and 4 the WAB rats received the appropriate infusion of saline or transfusion of blood. Fourteen days after the second infusion or transfusion (day 18) each animal received the tumour challenge subcutaneously in the left flank. The challenge dose was 1.0×10^6 viable cells. Daily tumour measurements were performed when the tumours were palpable. Fourteen days after tumour inoculation (day 32) the tumours were excised completely and weighed immediately. The flank wounds were sutured and the animals were maintained as described previously. After tumours had been excised the animals were bled as described above.

The PSA of each blood sample was measured against lymphocytes from healthy syngeneic rats and lymphocyte reactivity to PPD and a tumour extract was measured using the TEEM test (Section 2.3). The tumour extract

was prepared by mechanically slicing a single MC 7 sarcoma from a WAB rat, pressing the tumour slices through a wire sieve and collecting the tumour homogenate in Hank's BSS. The final concentration was made up to 1 x 10^7 cells/ml by adding BSS (Section 5.2.2). Tumour homogenate 0.1 ml (1 x 10^6 cells) was incubated with 0.5 x 10^6 lymphocytes from each animal in 3.0 ml BSS for 60 minutes at 23°C. The supernatant obtained after centrifugation was added to TSRBC indicator cells in the usual manner (Section 2.3.3).

The Student's t test was used for statistical analysis of the results of PSA and lymphocyte reactivity. The Wilcoxon rank sum test was used for statistical analysis of tumour volumes and weights.

5.4.2 RESULTS

5.4.2 (a) Plasma suppressive activity

The results have been plotted in Figure 5.3 as the log of the microlitre volume of plasma required to cause 50% inhibition of the response of normal syngeneic lymphocytes to PPD (Section 2.3.4) and the scale of the vertical axis has been inverted so that a rise in PSA (i.e. a decrease in the microlitre volume of plasma) is seen as a rise in the points plotted on the graph, and vise versa.

PSA was low in each group before infusion or transfusion (T). After the first transfusion of allogeneic blood there was a significant increase in PSA compared with the pre-transfusion PSA of the allogeneic group ($p<0.001$) and the PSA of the saline and syngeneic groups after their first infusion or transfusion (both $p<0.001$). There was a further increase in PSA after the second transfusion of allogeneic blood but PSA remained unchanged in animals given more syngeneic blood or saline. At the time of tumour inoculation (I), PSA in the allogeneic group was very high relative

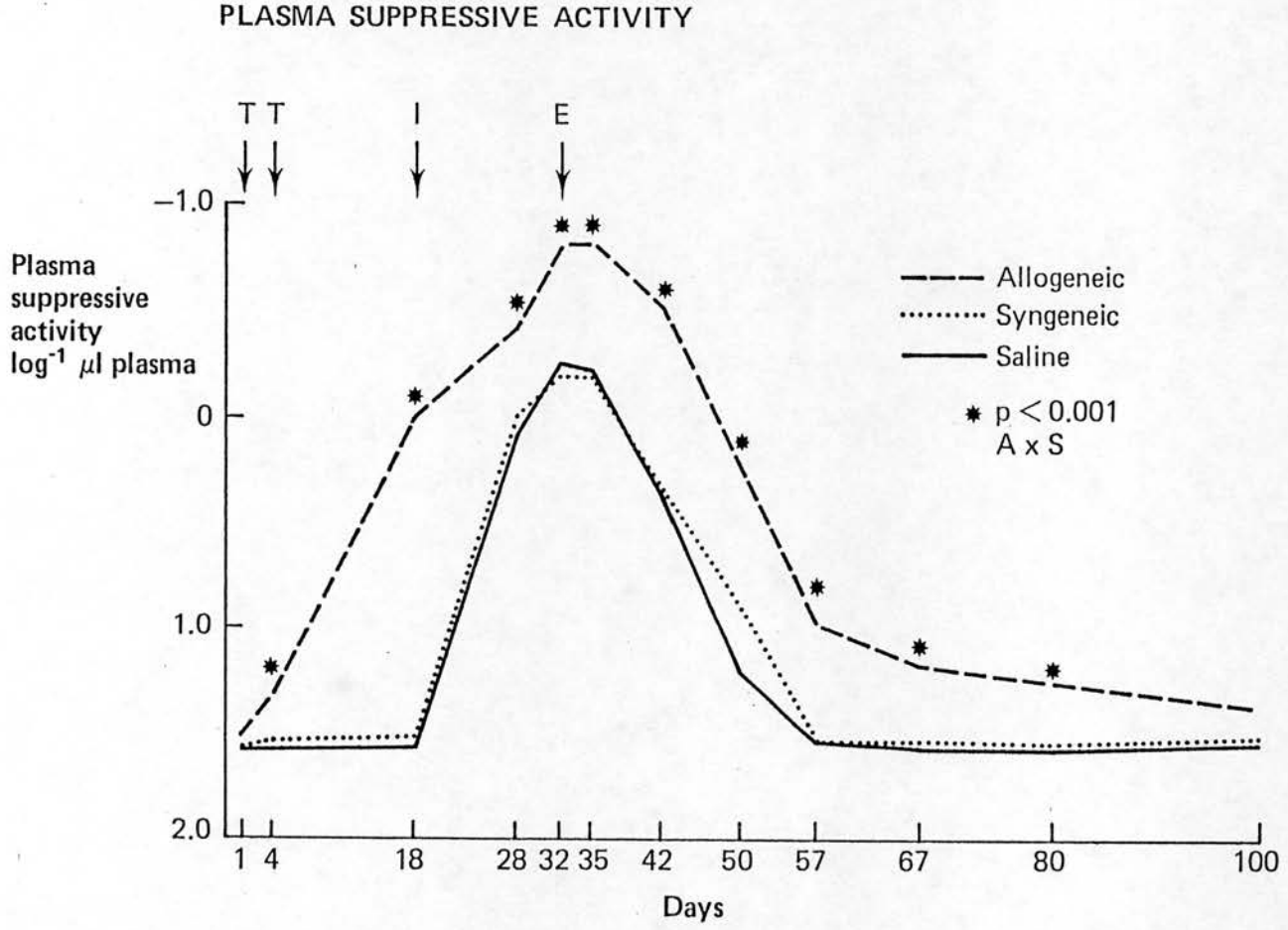


Figure 5.3 Plasma suppressive activities before and after infusion of saline or blood transfusion, and before, during and after tumour inoculation

T = Transfusion or infusion
I = Tumour inoculation
E = Tumour excision

to the other two groups. Following tumour inoculation, and concurrently with tumour growth, the PSA of all three groups increased but remained significantly higher in the allogeneic group. After complete excision of the tumours (E), PSA slowly returned towards pre-tumour levels: PSA returned to pre-tumour ("normal") levels by day 57 in the syngeneic and saline groups, that is 25 days after excision; PSA had been high before tumour inoculation in the allogeneic group and the pre-tumour level (or "day 18" level) was achieved before day 50. Return of PSA to normal or pre-transfusion levels in the allogeneic group was not achieved until at least 48 days after excision of the tumour, that is at some time between days 80 and 100 of the experiment.

5.4.2 (b) Lymphocyte reactivity

The results of <u>in vitro</u> lymphocyte responses to PPD stimulation are shown in Figure 5.4. On day 1, before any infusion or transfusion (T), the lymphocyte responses of the three groups to PPD stimulation were not significantly different. By day 4 the responses of rats given allogeneic blood had fallen significantly ($p<0.001$), whereas responses of the saline and syngeneic groups had not altered. There was a further fall in lymphocyte reactivity in the allogeneic group following the second transfusion but not in the other two groups. At the time of tumour inoculation (I) there was a highly significant difference ($p<0.001$) in lymphocyte reactivity to PPD between the allogeneic and the other two groups but no difference in the responses of lymphocytes from the syngeneic and saline groups. After tumour inoculation the responses of lymphocytes from all groups fell markedly. Immediately before tumour excision (E) the responses in all three groups were very low and were not significantly different. Following tumour excision lymphocyte reactivity returned

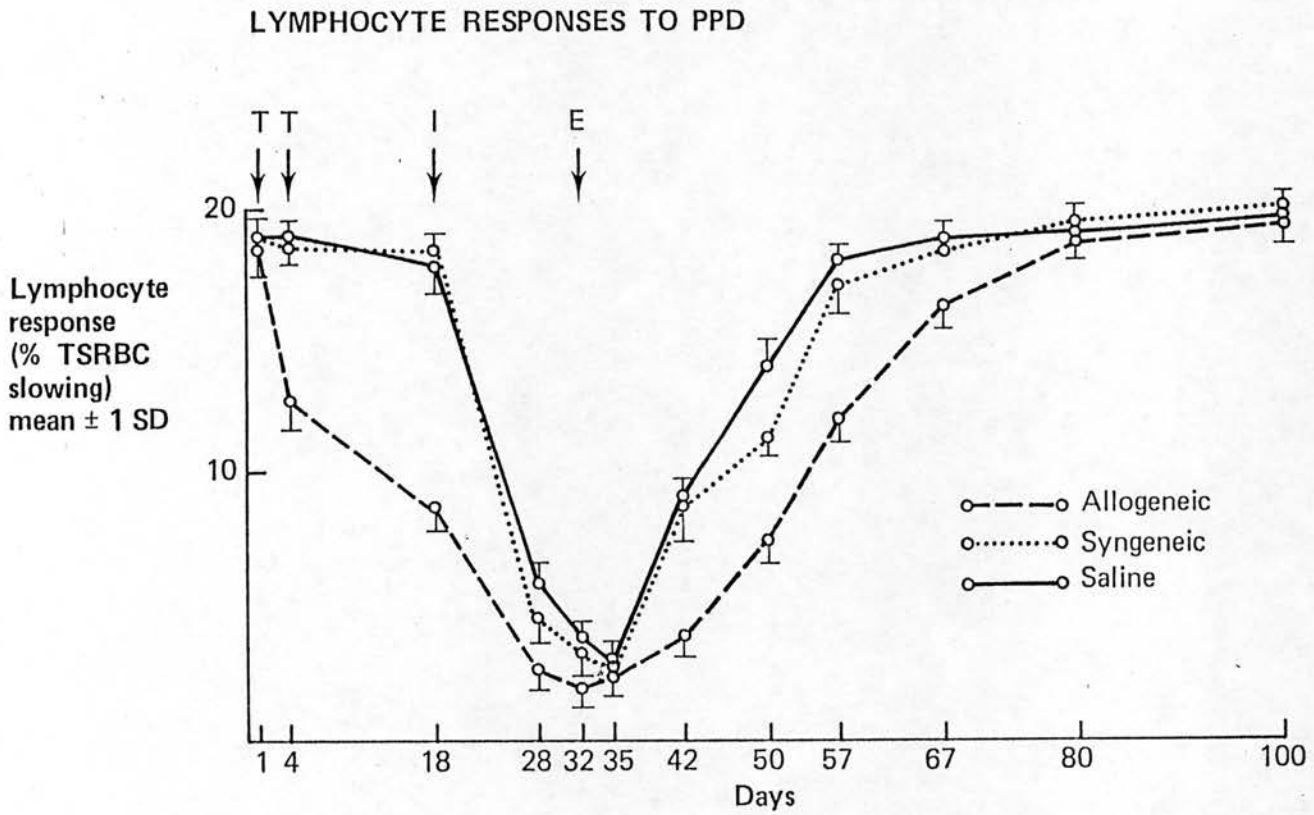


Figure 5.4 Lymphocyte responses to PPD before and after infusion of saline or blood transfusion, and before, during and after tumour inoculation

T = Transfusion or infusion
I = Tumour inoculation
E = Tumour excision

towards normal levels but at faster rates in the syngeneic and saline groups than in the allogeneic group. At day 67 the responses of lymphocytes to PPD of animals which received allogeneic blood were significantly lower than those of the other two groups ($p<0.01$) but the differences were not statistically significant by day 80.

Lymphocyte responses to tumour extract were measured immediately before tumour inoculation on day 18, immediately before tumour excision on day 32 and again 10 days later on day 42 (Table 5.3). Responses of lymphocytes from rats given saline were not statistically significantly different from those of the syngeneic group but were significantly higher than responses of animals given allogeneic blood. At the time of tumour excision lymphocytes in animals from all groups had become sensitised to tumour tissue, as indicated by the increase in *in vitro* reactivity to tumour extract. However, the responses of lymphocytes from animals which received an allogeneic transfusion were significantly lower than those of animals given saline ($p<0.05$) or syngeneic blood ($p<0.001$). Ten days after excision of the tumours the lymphocyte responses (mean ± 1SD) to tumour extract had fallen in the allogeneic group from 17.5% ± 0.3 to 12.3% ± 1.3, whereas reponses in the saline group had not decreased significantly (19.9% ± 1.9 to 18.4% ± 0.5).

The results indicate that *in vitro* lymphocyte reactivity measured by the TEEM test method was suppressed in the recipients of allogeneic blood but not syngeneic blood or saline. Tumour growth suppressed *in vitro* lymphocyte responses in all groups irrespective of pre-treatment, and when tumours were large (i.e. just prior to excision) the lymphocyte responses were uniformly low. Lymphocyte reactivity returned slowly to normal after the removal of the tumour. As these animals were in-bred laboratory strains it is unlikely that they had been exposed to Mycobacterium

Table 5.3 Lymphocyte responses to tumour extract expressed as percentage slowing (mean ± ISD) of TSRBC

	GROUP		
DAY	Saline	Syngeneic	Allogeneic
18	8.2 ± 1.0	8.6 ± 1.0	5.5 ± 0.8
32	19.9 ± 1.9	21.3 ± 0.5	17.5 ± 0.3
42	18.4 ± 0.5	-	12.3 ± 1.3

ALLOGENEIC x SALINE (Student's t test):

Day 18 : $p < 0.01$

Day 32 : $p < 0.05$

Day 42 : $p < 0.001$

tuberculosis and so would not have been sensitised to PPD which may have been stimulating the lymphocytes in a manner similar to that of a mitogen.

5.4.2 (c) Tumour volume

The mean daily volumes of tumours in animals with a significantly raised PSA at the time of tumour inoculation were significantly greater than the volumes of tumours in animals with a low PSA, from day 6 until day 14 when tumours were excised (Table 5.4). At the time of excision tumours in the high PSA ("allogeneic") group were nearly twice the size of tumours in the low PSA ("syngeneic" and "saline") groups. Tumours grew in all animals.

Growth rates of the tumours are plotted in Figure 5.5. At day 6 tumours in the low PSA groups were significantly smaller than tumours in the high PSA group. Before day 6 tumour growth in the low PSA groups was obviously less than in the high PSA group even though the tumours were too small for accurate measurement. However, after days 7 or 8 the growth lines of the three groups were almost parallel, indicating that after this time the pattern of growth of each group of tumours was approximately the same. This suggests that the implanted tumour cells were most susceptible to whatever host factors differed between the three groups during the first few days of their growth in the new host, and that as the tumours became established their growth became less influenced by host factors.

5.4.2 (d) Tumour weight

Tumours were excised completely on day 32, 14 days after inoculation, and were weighed immediately. There was no significant difference between the weights of tumours in the two low PSA groups

Table 5.4 Tumour volumes (mean ± 1SD)

Day after tumour passage	TUMOUR VOLUME (mm^3)			p VALUE*
	Saline	Syngeneic	Allogeneic	
6	44 ± 18	60 ± 19	354 ± 39	< 0.001
7	154 ± 23	178 ± 24	794 ± 124	< 0.001
8	257 ± 58	323 ± 70	1122 ± 230	< 0.01
9	398 ± 119	575 ± 101	1585 ± 360	< 0.02
10	691 ± 301	794 ± 136	2338 ± 570	< 0.02
11	954 ± 322	1175 ± 245	3290 ± 681	< 0.01
12	1513 ± 432	1798 ± 289	4786 ± 702	< 0.001
13	2511 ± 520	2794 ± 432	6625 ± 763	< 0.001
14	4012 ± 739	4786 ± 502	7752 ± 950	< 0.02

* Value of p for statistical comparison of allogeneic and syngeneic groups using the Wilcoxon two sample rank sum test.

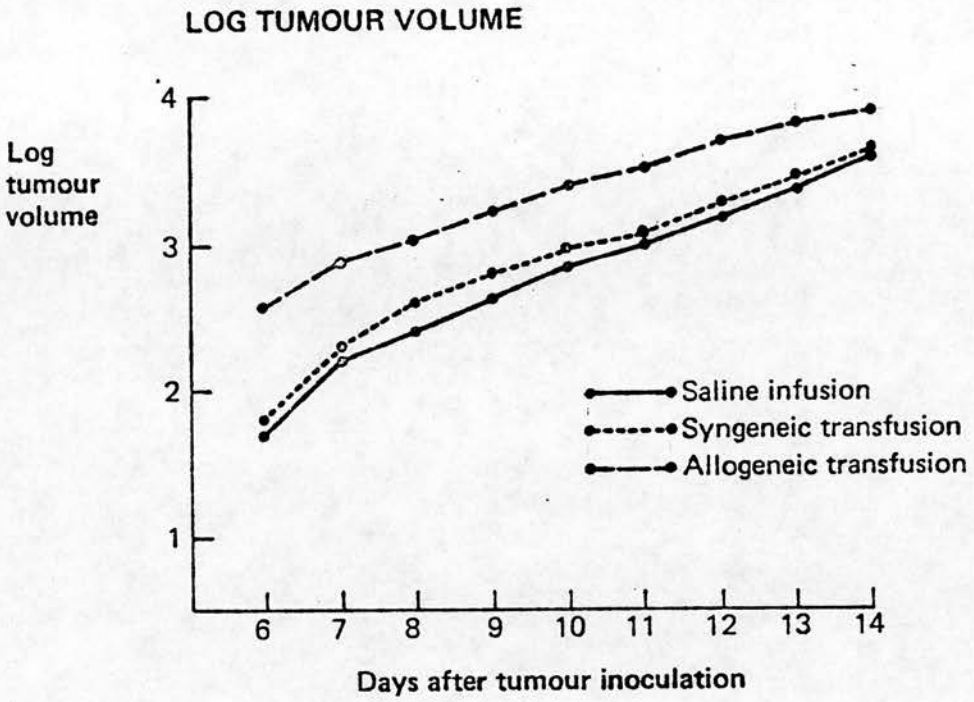


Figure 5.5 Mean tumour volumes (log mm^3)between the sixth and fourteenth days of growth.

(Table 5.5). However, tumours from the high PSA group were significantly heavier than those of the saline ($p<0.05$) and syngeneic ($p<0.05$) groups, which had low PSA values at the time of tumour transplantation.

5.4.3 DISCUSSION

This experiment examined the effect of manipulating PSA on the growth of a transplantable chemically-induced tumour in in-bred rats and found that transfusion of allogeneic blood, but not transfusion of syngeneic blood or infusion of saline, was associated with a significant rise in the lymphocyte suppressive activity of plasma. The use of normal syngeneic lymphocytes ensured that the apparent rise in PSA was due to activity in plasma and not inherently poor lymphocyte reactivity. Secondly, this experiment showed that tumour growth was associated with a significant increase in PSA irrespective of whether or not PSA was raised at the time of tumour inoculation. The rise in PSA was seen in all experimental groups and appeared to be additive in as much as there were further significant increases in PSA in animals in which PSA was already increased. Thirdly, it was shown that tumour growth was increased in animals in which PSA was greatly increased when the tumours were transplanted.

A high PSA was induced in WAB rats by transfusion of blood from allogeneic PVG/C rats. This method of PSA induction has advantages and disadvantages. The method is simple and readily reproducible, and the increase in PSA can be controlled accurately by administration of a known volume of blood. The experimental animals are spared the suffering of a disease process or tissue injury which might alter physiological and immunological functions in addition to increasing PSA. The major disadvantage of the blood transfusion technique is that whole blood

Table 5.5 Tumour weights

GROUP (n)	TUMOUR WEIGHT (grams) mean ± 1SD
Saline (12)	6.8 ± 2.1
Syngeneic (12)	7.0 ± 1.9
Allogeneic (12)	9.4 ± 3.2

ALLOGENEIC x SYNGENEIC: $p < 0.05$

(Wilcoxon two sample rank sum test)

consists of many cellular and humoral elements which have been shown recently to influence the immune system (Table 5.6). Although this experiment has demonstrated clearly that allogeneic transfusion leads to an increase in the lymphocyte SA of plasma in this animal model, it cannot be said that the increased PSA was the only operative mechanism which could have caused the increase in tumour growth. It is difficult to conceive of a direct method of increasing PSA without some accompanying unwanted physiological disturbance. The transfusion of plasma with high SA might have provided an indirect method.

The immunological effects of blood transfusion have been investigated extensively by researchers in the field of organ transplantation. A full review of this subject is beyond the scope of this work. However, it is apparent that allogeneic blood transfusions may cause specific (relative to the immunising antigens) and non-specific suppression of some immune functions. In an attempt to explain the mechanism or mechanisms by which blood transfusion benefits subsequent renal allograft survival, Van Rood[388] has suggested that immunisation of an individual with allogeneic blood induces suppressor cells which often, but not always, suppress the formation of anti-HLA antibodies and possibly cytotoxic lymphocytes, depending on the presence of appropriate Ir genes. This suppressed state allows the formation of anti-idiotype antibodies which, in turn, inactivate T cell clones which would have reacted against HLA antigens present on the immunising cells of the allogeneic blood. If the blood transfusion donor and a subsequent tissue allograft share antigens, then the inactivation of the responding clones is optimal. For example, tumour growth would be accelerated in the case of a tumour allograft and rejection of a kidney would be impaired in the case of a renal allograft.

Although tumours are known to bear surface antigens which may also

Table 5.6 Immunological consequences of allogeneic blood transfusion (from Francis et al[217])

1. Induction of HLA-independent non-specific suppressor T-lymphocytes (Lenhard et al [375]).
2. Induction of antigen specific suppressor T-lymphocytes (Marquet et al [376]).
3. Increased suppressor cell activity (Kerman et al [377]).
4. Reduced natural killer cell activity (Singal et al [378]).
5. Impaired mononuclear phagocytic cell function (Keown et al [379]).
6. Depletion of phagocytic cells (Van der Linden [380]).
7. Induction of immune complexes (Benzonna et al [381]).
8. Induction of lymphocyte suppressive activity in plasma (Shenton et al [344] and Francis et al [382]) associated with alpha-2-macroglobulin (Alomran et al [383]).
9. Increased numbers of circulating IgG-, IgM- and IgA- secreting cells (Gascon et al [384]).
10. Induction of non-cytotoxic anti-Fc receptor antibodies (Macleod et al [385]).
11. Induction of anti-idiotypic antibodies (Singal et al [386]).
12. Induction of donor specific lymphocytoxic antibodies (Ettenger et al [387]).

be found on normal tissues of the adult host, they also bear neo-antigens which may be unique to the tumour cells (Section 1.4.1). In particular, chemically-induced tumours have unique antigens which may not even exhibit cross-reactivity with tumours of identical histological appearance induced by the same carcinogen in a syngeneic animal. Thus, while the host may be rendered specifically unresponsive to antigens shared between the immunising blood and the tumour, alternative non-specific mechanisms have to be proposed to explain how allogeneic blood transfusion allows an accelerated rate of growth of a highly immunogenic tumour bearing unique surface antigens. One such mechanism is the rise in PSA which accompanies allogeneic transfusion in humans[344,389], and experimental animals (as demonstrated in the present study).

It has been demonstrated that the transfusion of allogeneic blood increases the immuno-regulatory activity of A2M and that this naturally occurring globulin acts non-specifically to suppress immune responses of lymphocytes[374]. A2M has wide ranging immunoregulatory activities against several types of cells (Section 1.5.8). It is conceivable that the "blood transfusion effect" demonstrated in this study acts in part by non-specific suppression of immune activities, including those of lymphocytes.

In spite of the limitations imposed by the use of allogeneic blood transfusion to increase PSA, the findings are of considerable interest. It has been shown that both tumour growth and transfusion of allogeneic blood caused a rise in PSA and that these effects were additive. Even when the tumours in all three groups were large, the PSA of the syngeneic and saline groups of rats were not as great as that of the allogeneic group. Also, the PSA returned to pre-tumour levels after excision of the tumours, a finding which could be exploited perhaps in the area of clinical oncology.

Perhaps the most significant observation in this experiment is that

allogeneic blood increased the growth of a transplantable chemically induced tumour, a finding which is incidential to the aim of the experiment. The importance of this observation is potentially immense and is being investigated currently by the author. Since the present experimental work was performed and published[382,390], there has been considerable interest in the effect of blood transfusion and tumour growth in humans and experimental animals. The subject has been reviewed recently by the author[217, 391], although further discussion is beyond the scope of this thesis.

5.5 DISCUSSION

The first experiment reported in this chapter has shown that a causal relationship exists between the growth of a tumour and a rise in the SA in the host's plasma (Section 5.3). Unfortunately, time did not allow the author to analyse the plasma with increased SA to determine the responsible substance or substances in the experimental rats. However, studies described in Chapter 3 indicate that A2M is largely responsible for the increase in PSA in humans with tumours.

Several naturally occurring immunosuppressive substances have been described in patients with malignant disease (Section 1.5.7). Sequential studies of a few of these factors[392-394] and other "tumour markers", such as carcino-embryonic antigen[395], human chorionic gonadotrophin[396] and acute phase reactant proteins[397], have indicated that their serial measurements may be of value as prognostic indicators following cytoreductive therapy. However, in many cases poor specificity and sensitivity have limited the use of such single or multiple marker assays in clinical practice. An alternative approach would be to measure

sequentially the immunosuppressive activity in plasma rather than the concentrations of individual biochemical markers. In contrast to biochemical assays, measurement of PSA represents a functional assessment of at least some aspects of immune function. The present study provides support for the measurement of PSA in longitudinal studies in other tumour models and, ultimately, in humans.

The close correlation between increased PSA and tumour growth in rats with primary tumours and in the single case of tumour metastases supports the hypothesis that the high PSA in tumour-bearing subjects is a direct consequence of the presence of tumour tissue. The relatively slow return of PSA to pre-tumour levels after tumour excision may reflect the slow resolution of factors responsible for the high PSA. There may be several reasons. Firstly, the human studies indicate that the high PSA in tumour-bearing subjects is due to several factors and, for example, while the electrophoretically-fast form of A2M may be eliminated rapidly immune complexes may be removed at a much slower rate. Secondly, surgery and anaesthesia have an immunosuppressive effect, as shown by the reduced lymphocyte responses of patients receiving only crystalloid solutions in the intravenous fluid study (Section 4.3), and this effect may prolong the impairment of immune reactivity associated with tumours. Thirdly, it is possible that the high PSA detected after complete excision of the tumours was not related directly to the tumour or tumour products but rather was related to altered metabolic or nutritional states induced by the tumour. Some support for this view is provided by the studies reported in Section 3.5.3 in which an increased PSA was found in patients suffering from malnutrition unrelated to malignancy. Fourthly, it is possible that the tumours were not removed completely, as would occur with complete local excision in the presence of distant metastases; however, this scenario is

unlikely because the PSA of the single animal which developed metastases behaved initially in precisely the same manner as that of the other rats rather than remaining elevated and being unaltered by tumour excision.

The second experiment (Section 5.4) showed that transfusion of allogeneic, but not syngeneic, blood produced a significant depression in lymphocyte reactivity to PPD and a significant increase in PSA, as measured by the TEEM test, and so provides further evidence of the immuno-modulating effects of transfusion (Table 5.6). The volume of each of the two transfusions was 2.0 ml which represents 10 - 15% of the circulating volume of the rat. The experiment suggested, but did not prove, that the increase in PSA was beneficial to tumour growth; the method of inducing a high PSA (transfusion) would have resulted in changes in other immunological parameters which could have also altered tumour growth. However, the experimental results were consistent with the hypothesis that PSA is beneficial for tumour growth.

5.6 SUMMARY

The lymphocyte SA of plasma was measured sequentially in tumour-bearing rats and was correlated with tumour behaviour. Twelve female adult WAB rats were inoculated with a methylcholanthrene-induced sarcoma from a syngeneic animal and bled before, during and after tumour growth. The PSA increased rapidly with tumour growth and declined slowly following tumour excision. In animals remaining free of tumour PSA remained at pre-tumour levels. PSA rose with the occurrence of metastases. The study demonstrates that PSA closely parallels the growth of primary tumours (as well as the growth of metastases in a single case), and gives support to the sequential measurement of PSA in subjects treated for malignant

disease.

Allogeneic transfusion was associated with significant depression of *in vitro* lymphocyte reactivity to PPD and tumour extract, and a significant increase in PSA, whereas saline infusions and syngeneic blood were not. Tumour volume and weight were significantly greater in animals with a high PSA and low lymphocyte reactivity than in animals with relatively normal PSA and lymphocyte reactivity at the time of tumour transplantation, suggesting that a high PSA, and impaired lymphocyte reactivity, favoured tumour growth.

CHAPTER 6

DISCUSSION

Many aspects of the work have been discussed within the various sections of the thesis. However, some points concerning the methods and results require discussion within the overall context of the reviewed literature.

6.1 METHODS

6.1.1 THE TEEM TEST

The study aimed to examine firstly the intrinsic ability of lymphocytes to respond to standardised stimuli, and, secondly the capacity of plasma and individual plasma components to inhibit lymphocyte reactivity, and therefore it was necessary to study separate components of CMI under controlled conditions and to be able to quantify the results. In particular, it was necessary to assess objectively lymphocyte reactivity in an environment free of humoral factors. This excluded the use of *in vivo* tests (such as CDHR) and *in vitro* tests which require human or xenogeneic serum. The problems of standardising and interpreting *in vivo* tests of CMI have been discussed (Section 2.3).

The TEEM test was chosen as an *in vitro* method of quantitatively assessing lymphocyte reactivity and the suppressive capacity of plasma and plasma factors. The TEEM test has superceded earlier electrophoretic tests of CMI, such as the MEM and MOD-MEM tests (Section 2.3.1), and many of the problems of these older electrophoretic tests have been eliminated by the use of tanned sheep erythrocytes as indicator particles in the TEEM test. These cells are inherently stable with respect to their electrophoretic mobility, and their method of preparation is straight forward. It is important to emphasise that the TEEM test is a method of detecting and measuring lymphokine release from lymphocytes in response to antigens or mitogens; it is not a test for the presence of malignant disease, as has

been suggested by some proponents of the older electrophoretic tests.

The technique of cytopherometry is very demanding and requires considerable skill on the part of the operator. A criticism of electrophoretic tests is that they are open to operator bias and subjective interpretation of their results. Consequently, all of the cytopherometry was undertaken by Dr. Brian Shenton, an internationally recognised expert in the theory and practise of cytopherometry. Cytopherometry specimens were coded by the author before being examined by Dr. Shenton and the code was broken only after the TEEM test had been performed and the results secured by the author. In this way, samples were tested independently by a skilled worker in the field without knowledge of their source.

The TEEM test has been found to be highly reproducible. Studies of reproducibility have been undertaken in our laboratory and have been reported (British Society of Immunology, London, April 1983). The TEEM test yields consistent results over a period of time: for example, during the seven month period of August 1980 to February 1981 the author donated lymphocytes for control studies on 27 occasions and reactivity to PPD was measured; during this time the author remained in good health and received no medications which could have altered lymphocyte reactivity. Reactivity was measured as percentage slowing of TSRBC (Section 2.3.3(d)) and was 18.6% ± 1.4 (mean ± ISD) with a range of 16.1 to 20.7%.

Comparative studies show that the TEEM test correlates well with the MEM test[327] and the lymphocyte transformation assay[328]. Also, studies[398] performed in the laboratory have investigated dose-responses of normal human lymphocytes to the immunosuppressive effects of hydrocortisone, methyl prednisolone and cyclosporin A, and results with the TEEM test have been reproducible and have agreed with those of others[399].

The advantage of the TEEM test over more traditional in vitro methods of assessing lymphocyte reactivity is that it takes only four hours to perform, thereby allowing assessment of very large numbers of samples within a relatively short time. This was an essential feature of the study as several thousand samples of plasma, plasma fractions and lymphocytes were assayed.

Currie et al[126] showed that washing lymphocytes from cancer patients enhanced their specific in vitro cytotoxicity against autologous and allogeneic tumour cells and believed that the effect was due to removal of an "inhibitory serum component" from the lymphocyte surface. Thus, preparation of lymphocytes for this study was meticulous and strictly standardised to remove this important source of error. Previous studies by Shenton et al[327] have investigated the effects of washing lymphocytes for use in the TEEM test, and the method of preparation used in the present study was the same as that of Shenton et al[341,344].

6.1.2 SUBJECT GROUPS

It was decided before commencing the project to study patients with malignant disease of the gastro-intestinal tract and breast. Patients were typical cases presenting to a general surgical unit for investigation and treatment. A small group of entirely healthy individuals acted as controls, being selected only on account of their good health and availability. A second control group of patients with benign disease was selected on the basis of age, sex and the site of their disease, with the aim of eliminating some of the obvious differences between the malignant and healthy groups. The limitations of such selection are acknowledged. It is clearly not possible to "match" two disease processes when one is malignant and the other not, but it is also clear that variables such as

nutritional status and age can affect immune performance and so attempts were made to eliminate such variables wherever possible within the limitations of the available patient population. Comparison of Tables 2.1 and 2.2 indicates that selection of benign patients at times failed to provide patients with diseases of similar tissues or organs as the malignant group. However, in many cases the patients' symptoms suggested that their inclusion in the benign "control" group had clinical relevance.

6.1.3 MALNUTRITION

6.1.3 (a) Measurement

Methods of assessing nutritional status have been discussed (Section 1.6.1). It is clear that no single method is satisfactory in terms of sensitivity, specificity or availability. The most accurate method is measurement of body composition by TBNAA, a technique which was not available to the author for this study. Therefore, a number of widely used and well tried methods of assessment were selected, rather than relying on any single method. The justification for using weight-for-height, weight loss, arm muscle circumference, triceps skin fold thickness and serum albumin concentrations is that firstly, these parameters have been regarded as corner-stones of clinical nutritional assessment protocols for many years and are used regularly by clinical nutritionalists in hospitals throughout the world; the five parameters are useful in clinical practice and were the standard methods of assessment within the hospital where the study was performed. They are well accepted nutritional parameters. Secondly, these parameters correlate well with body composition studies using TBNAA, and although anthropometric and biochemical assays lack the sensitivity of TBNAA it is reassuring that highly significant and

reasonably strong correlations have been demonstrated[232]. Nutritional parameters become clinically relevant only when they are able to detect patients who are at risk of serious complications if their nutrition is not improved and who will benefit from nutritional therapy. The literature indicates that the above parameters are indeed clinically relevant (Section 1.6.2), and therefore they were used in the present study.

6.1.3 (b) Standard nutritional data

Patients with nutritional abnormalities are identified by comparing their nutritional data (anthropometric, biochemical or functional) with that of normal or 'standard' populations. Thus, "malnutrition" is a relative definition. The standard population must be relevant to the group of subjects being assessed, otherwise the sensitivity of the classification or comparison is open to question. However, standard data were not available for the local population from which the experimental subjects came. There have been no epidemiological studies of nutritional status of the adult population of the north-east of England and so alternative "standard data" had to be used. The standard data used by clinical nutritionalists at the Royal Victoria Infirmary, Newcastle-upon-Tyne, at the time of the study were the Metropolitan Life Insurance Company tables[400] which have been used widely in nutritional assessement[230,238], and these were adopted by the author.

The use of these tables was not ideal and is open to criticism. The Metropolitan Life Insurance Company created body weight standards in 1943 by surveying North American populations, and they were referred to initially as tables of "ideal weight". They were revised in 1959 and referred to as "desirable weight" tables for each of three 'frame' types (small, medium or large), following the realisation that subjects varied in

skeletal build and that the skeleton made a substantial contribution to body weight. Measurements of frame type were not made. The inclusion of body frame type was a considerable advance on the original tables, even though only three broad categories were included. It has to be accepted, however, that because of the absence of definitions of frame type[401] comparisons of individuals with standard data may lead to inaccuracies in defining underweight subjects: being below an abstract average value does not necessarily signify that body weight is below "normal" or lower than it should be for a person's body build, age, sex, height or general state of health. It was for this reason that '85% of normal' was used as the point of identification of undernutrition, so that there would be some lee-way in the definition of malnutrition to account for variations in body type.

It has been recommended that tables of "ideal" or "desirable" weights-for-height should be replaced by skin fold thickness measurements appropriate to each body type[402,403]. As stated above, there is still no satisfactory definition of various body builds, although most investigators can subjectively categorise most subjects. It is, therefore, inappropriate to rely on just one method of nutritional assessement, such as body weight-for-height, and so five criteria were used in the present study. There is, of course, no way of avoiding the use of normal standard values; "abnormal" is entirely dependent on "normal".

All so-called 'standard data' are open to these criticisms, irrespective of whether they are derived from North American or local populations. Ideally, a nutritional survey of the local population should have provided the standard data, but that was beyond the scope of the work. (It could perhaps be argued on sociological grounds that the standards obtained from such a survey of the north-east of England at the time when

the experimental work was performed would have been closer to the Metropolitan Life tables than to present day North American standards). Support for the contention that the North American data of 1959 is not greatly different from the local experience comes from the fact that none of the normal healthy volunteers in the study could be classified as undernourished by any of the nutritional parameters. The inclusion of this group of subjects is important, therefore, not only because of the experimental findings of lymphocyte reactivity, PSA and A2M, but also because the results of their nutrition assessments add validity to the use of the Metropolitan Life tables as a source of normal values in the absence of standards from the local population.

The advantages and disadvantages of functional tests, such as hand grip dynamometry, have been discussed (Section 1.6.2(d)). As with anthropometry, standards are required for functional methods of assessment, but comparative standard tables were not available for the population from which the experimental subjects came.

6.1.3 (c) Definition

The point of distinction between normal and abnormally low nutritional measurements was taken as less than 85% of standard values for anthropometric measurements and less than two standard deviations below the mean for serum albumin concentrations. As expected from the nature of methods of assessment and the standard values employed, not all patients had all five parameters above or below the 85% cut-off point: 62% had less than two abnormal parameters, 5% had two, and 33% had more than two. Therefore, it was decided to classify patients as malnourished if the majority of their parameters were "abnormal" as defined above. The distribution of normal and abnormal results indicates that there was fairly

close agreement between the discriminatory powers of the five parameters based on the 85% cut-off mark. Others[244,404,405] have also used the 85% cut-off point to define malnourished individuals.

6.2 LYMPHOCYTE REACTIVITY

The complex relationship between nutrition, cancer and immunological reactivity has been explored by the experiments reported in Sections 3.2 and 3.3. Lymphocyte reactivity to a recall antigen and to mitogens, as assessed by an electrophoretic technique, was significantly lower in well nourished cancer patients than in well nourished patients with benign conditions or in healthy subjects. Malnutrition adversely affected lymphocyte reactivity significantly, and malnourished cancer patients generally had significantly lower lymphocyte responses than malnourished benign patients.

The results indicate that malnutrition and malignancy both impair lymphocyte reactivity and that unlike PSA, their effects are cumulative. Therefore, any investigation of lymphocyte function in cancer patients must take account of nutritional status. Although it is difficult to distinguish cause and effect in the relationship between nutrition, cancer and impaired immuno-competence, it is clear that malnutrition cannot be ignored as a cause of immunoparesis in cancer patients. Therefore, studies which correlate the outcome of cancer treatments with tests of immune competence may merely reflect nutritional consequences of disease or its treatment rather than any direct effect of therapy itself.

What is the significance of impaired lymphocyte responsiveness? The reviewed literature emphasises the central role of lymphocytes in the afferent and efferent limbs of specific immune responses, and so, by

implication, the immune response against cells exhibiting tumour antigens would be reduced and tumours would be subjected to weaker immunological attack if lymphocyte function was impaired. Support for this supposition has been provided by the animal study in which reduced lymphocyte responses to PPD, PHA and a tumour extract were associated with increased tumour growth. Also, circumstantial evidence from clinical transplantation suggests that impairment of immune function is associated with rapid and aggressive tumour growth[406]. The mechanisms responsible for impairment of function of lymphocytes and other immune cells are many and varied (Section 1.5), and a further mechanism has been proposed in this thesis (Section 1.8).

The use of tests of immune function in assessing malnourished patients is not accepted generally and the literature concerning their value is contradictory (Section 1.6.2 (e)). This study showed strong significant correlations between in vitro lymphocyte reactivity and the five nutritional parameters in patients with benign disease (Section 3.2.2), suggesting that lymphocyte reactivity as measured here is a valuable indicator of malnutrition in these patients. The results in patients with malignancy were different: correlations between lymphocyte reactivity and nutritional parameters were generally weaker and 30% were not statistically significant, indicating, firstly, that lymphocyte reactivity was a poor index of malnutrition as measured by the five criteria in these cancer patients, and, secondly, that factors other than cancer were affecting lymphocyte reactivity adversely. Therefore, it is reasonable to recommend the use of in vitro lymphocyte reactivity to PPD and mitogens as a correlate of nutritional status in patients in whom there are no other factors which adversely affect lymphocyte function.

6.3 PLASMA SUPPRESSIVE ACTIVITY (PSA)

The study has shown that SA in plasma from cancer patients can be measured quantitatively by a simple, rapid *in vitro* technique, a significant achievement of potential importance in two ways. First, sequential studies of tumour markers (such as carcino-embryonic antigen and human chorionic gonadotrophin) indicate that their serial measurement in some patients is of prognostic significance, although poor specificity and sensitivity have limited their widespread use in clinical practice. An alternative approach would be to measure sequentially the immunosuppressive activity in plasma rather than concentrations of biochemical markers. Support for this proposal comes from the animal study (Chapter 5) in which not only was PSA related to tumour growth but PSA returned slowly to pre-tumour levels following complete tumour removal. Also, PSA was very high in all cancer patients and, unlike PSA in benign patients, was not influenced by nutritional status. In contrast to biochemical assays, measurement of SA represents a functional assessment of immune status, and, like assays of tumour markers, PSA also represents indirectly a 'tumour product', as the mechanism of production of an elevated PSA is through the interaction of tumour products (proteases, antigens) with the host's response to the tumour (A2M, proteases from immune and inflammatory cells, antibodies). Therefore, serial measurements of PSA in patients treated for malignancy may be of value as indices of recurrent or residual disease.

Second, patients with malignancy were distinguishable from healthy subjects and from benign well nourished patients on the basis of the ability of their plasma to suppress the activity of allogeneic lymphocytes (Figure 3.18). This interesting observation has to be interpreted with caution. The malignant and healthy volunteer groups were not well matched and the numbers in each were small. PSA is non-specific in that it results

largely from the indiscriminate uptake and inhibition of virtually all classes of protease enzymes by a ubiquitous "scavenger" molecule, and therefore may be elevated by any condition associated with the release or production of proteases (e.g. ureamia or blood transfusion[374]). The use of this assay in clinical practice to identify patients with malignancy could be fraught with problems related to normal values, the exclusion of perhaps many patients because of other medical problems, and falsely low values in patients with early tumours. Clearly, many interim studies would be required before estimation of PSA could be used clinically.

6.3.1 PLASMA SUPPRESSIVE ACTIVITY AND MALNUTRITION

An aim of the present study was to determine whether a correlation existed between PSA and nutrition parameters (Section 3.5.3). Strong correlations existed between PSA and all five nutritional parameters in patients with benign diseases (Table 3.12 and Figures 3.19 and 3.20). The strongest correlation of PSA against autologous lymphocytes was with serum albumin concentration ($r = 0.83$, $p < 0.001$). The value of autologous PSA is a reflection of the capacity of a patient's plasma to inhibit the reactivity of his own lymphocytes; if lymphocytes are intrinsically suppressed as a result of malnutrition then PSA will be elevated falsely. The autologous PSA, therefore, is a compounded value, representing the suppressive capacity of plasma and reactivity of lymphocytes. This latter factor is removed by the use of lymphocytes from healthy allogeneic donors which allows measurement of the plasma's SA alone (allogeneic PSA). The strongest correlation of allogeneic PSA was with TSF thickness ($r = 0.84$, $p < 0.001$).

Correlation of PSA with nutritional status is a new finding which

supports the use of the five nutritional indices and provides another nutritional index which can be regarded as a test of immune function. Measurement of PSA in malnourished patients serves not only to help classify a patient's nutritional status but also to assess the function of the immune system. The other abnormalities of immune function secondary to malnutrition, and the advantages of functional tests over static methods of nutritional assessment, have been discussed earlier (Section 1.6.2). The mechanism of the elevation of PSA in malnourished patients is discussed elsewhere (Section 6.5).

No association existed between PSA and nutritional status in patients with malignant diseases although strong correlations were identified in the benign group. Failure to find an association in malignant patients may be related to the mechanism by which the high PSA was produced in these patients. A2M was the predominant factor associated with PSA; highly suppressive complexes are produced by saturation of A2M with proteases, and cancer cells and immune cells are plentiful sources of protease. If the saturation of A2M and removal of A2M-P complexes is as fast as the turnover of A2M (i.e., the rate at which complexed A2M can be replaced by free A2M) then the A2M mechanism responsible for producing the high PSA is maximal and cannot be increased further by production of more proteases. Of course, PSA could be increased further by way of the other mechanisms identified by this study or by entirely new mechanisms. It is known from the findings of the benign group that malnutrition does increase PSA, and so the failure to find an association between nutrition and PSA in patients with very high PSA values is consistent with the view that the mechanism by which malnutrition produces a high PSA (namely, A2M and small MW peptides) was saturated in the malignant patients.

6.4 ALPHA-2-MACROGLOBULIN AND MALNUTRITION

Immunosuppressive activity was associated with A2M and small MW peptides in malnourished patients with benign disease. The amount of SA associated with A2M in these patients was less than that in well nourished and malnourished cancer patients but greater than that in normal subjects and well nourished patients with benign disorders (Table 3.25). These are new findings which provide, in part, an explanation of the mechanism of impaired immunocompetence so often recognised in acutely malnourished individuals.

Malnourished patients with benign disease (benign malnourished, BMN) suffered mainly from conditions with an inflammatory component causing release of proteases at local tissue sites. Malnutrition is characterised by increased muscle (visceral protein) breakdown which provides another source of protease enzymes. Similarly, unrecognised infection increases catabolism, stimulates immune responses and leads to tissue necrosis and further inflammatory reaction. Unfortunately, it is not possible in this study to separate effects of malnutrition alone from those of inflammation, albeit localised, because the underlying cause of malnutrition in these patients, and indeed virtually all hospitalised patients in our society, is a disease process. Thus, it is not possible to say categorically that increases in SA associated with A2M and the small MW peptide were due to tissue catabolism alone. Three of the five patients in the benign well nourished (BWN) group studied in detail had conditions associated with inflammation (diverticular disease (2) and duodenal ulcer (1)) but none had raised PSA. These BWN patients are not adequate controls for the BMN patients who in all probability had at least mild inflammation associated with their disease (certainly those with ulcerative colitis (2) and chronic pancreatitis). There is no satisfactory method of staging or quantifying

the inflammatory process and it may be that the finding of a raised PSA in the BMN group was in fact an index of the degree of associated inflammation rather than a reflection of the malnourished state. Therefore, the results have to be interpreted cautiously, and although the increase in PSA and altered biological behaviour of A2M may be due to the metabolic disturbance in these patients, the possible role of the inflammatory process should not be forgotten.

The finding of a small MW peptide fraction with suppressive activity in malnourished subjects is a new finding; its association with A2M which had increased SA supports the proposal made by Hubbard[208] in relation to cancer (Section 1.5.8). Less SA was associated with A2M in the BMN group than in the malignant group; similarly, less SA was associated with the small MW peptides from BMN patients than peptides from cancer patients. However, ratios of SA in region 3 : region 6 after IgG-Fc depletion were similar in the three groups (Table 6.1), an observation which supports the hypothesis that there is an association between the peptide and A2M. This apparent relationship of function says nothing about a quantitative relationship. It is noted that the ratios are not precisely the same; differences may simply reflect the small numbers in each group. Also, it may be incorrect to assume that all SA in region 6 after Fc depletion was due to the cleavage product of A2M; other molecules, perhaps released from tumours or tissue catabolism, may have SA and may increase or decrease the SA assumed to be associated with peptides. However, the finding that the ratios of SA in region 3 and 6 are similar could be interpreted as indicating that there is a constant relationship between the SA of A2M and the peptide fraction, with A2M having much greater SA.

Table 6.1 **Suppressive activity (median and range, μl plasma) associated with A2M and the small molecular weight peptide fraction after IgG-Fc depletion.**

	SUPPRESSIVE ACTIVITY (μl plasma) (median, range)		
	A2M	Peptide	Ratio
BMN (n=5)	4.5 (4.2 - 35.8)	62.5 (39.6 - 78.4)	1 : 14
MWN (n=6)	1.6 (1.1 - 2.2)	37.3 (10.4 - 43.8)	1 : 23
MMN (n=6)	2.1 (1.2 - 4.8)	38.7 (33.2 - 59.9)	1 : 18

BMN = Benign malnourished group

MWN = Malignant well nourished group

MMN = Malignant malnourished group

6.5 IMMUNOLOGICAL ESCAPE AND CIRCULATING NON-SPECIFIC IMMUNOSUPPRESSIVE FACTORS

A paradox exists within the relationship between a tumour and its human host. On one hand there is clear evidence of specific immunological reactions by the host against his tumour, and, although immune reactivity in many cancer patients is depressed, it is not entirely paralysed. On the other hand, tumours which have reached the stage of clinical detection are able to grow and disseminate, even in the presence of specific immunological detection and attack, and only very rarely is there evidence of tumour regression in the absence of cytoreductive therapy. The recognition of this paradox has led to the concept of immunological escape of tumours. Several mechanisms exist by which malignant cells are able to evade immune destruction (Section 1.5): they include host factors, such as genetic restriction of host immune responses, and the existence of immunologically inaccessible sites, and factors related to the tumour, including changes in cell surface antigens to prevent immune recognition and destruction, shedding of surface antigens so that host antibodies combine with free antigen rather than antigenic components of tumour cells, and shedding of surface protease enzymes which can destroy host antibodies and facilitate growth and spread of malignant cell lines. The role of Ts lymphocytes as a mechanism of immunological escape is uncertain (Section 1.3.2). Antibodies formed in response to tumour antigens have been shown to 'protect' tumours in some respects by stimulating anti-idiotypic antibodies and masking antigenic specificities after combining with determinants on the cell surface.

Detection of circulating non-specific immunosuppressive factors (CN-SIF) in cancer patients suggests yet another mechanism by which tumours can escape immune destruction. Studies have indicated that the function of

many immune cells is impaired in some cancer patients but it has not been ascertained whether impairment is due to intrinsic defects of immune cells or the existence of circulating immunosuppressive factors. The present study has shown that lymphocytes from cancer patients had significant impairment of their in vitro reactivity in comparison with those from healthy subjects or patients with benign disease, and that plasma from cancer patients had highly significant increases in SA. The increase in SA was largely but not entirely non-specific in nature in that it was directed against autologous and healthy allogeneic lymphocytes and inhibited the responses of lymphocytes to a recall antigen (PPD) and three mitogens (PHA, Con A and PWM). The study indicates clearly that CN-SIF are important in the overall immunological deficit observed in cancer patients.

Unlike other studies of CN-SIF, the present work not only found that SA in cancer patients was associated with immune complexes (IC), alpha-2-macroglobulin (A2M), IgG (SA against allogeneic cells only) and Fc fragments of IgG, but was able to measure PSA quantitatively with the result that A2M was identified as the major inhibitory factor, while Fc fragments had slightly higher SA than IC. Other small MW substances (as yet undefined) were shown to be suppressive also. The importance of this last group of inhibitory substances is unknown, but the possibility arises that they may be analogous to the so called "middle molecules" detected in chronic renal failure patients and may result from the shedding and catabolism of membrane and intracellular components of abnormal neoplastic cells. They may include substances derived from the alpha globulins as observed by Glasgow et al[163] and Friedman et al[164] or from A2M as postulated by Hubbard[208].

6.5.1 ALPHA-2-MACROGLOBULIN

The present study identified the protease inhibitor A2M as a substance with lymphocyte SA in all subjects. The biological behaviour of A2M was different in patients with malignant disease in that A2M was far more suppressive than in normal subjects (Section 3.8.3).

The work suggests a new mechanism of immunological escape involving A2M, and provides further information about the activity of A2M in cancer. The following model, which is compatible with previous studies of the structure and function of A2M, is proposed. Tumour cells express a variety of surface determinants which lead to antibody and cell-mediated immune responses and a local inflammatory reaction as part of the immune response following release of lymphokines and other substances from lymphocytes, macrophages and polymorphonuclear leucocytes. Many of these substances (histamine, kinins, prostaglandins, complement components, lysosomal enzymes) depend on protease enzymes for their synthesis, release and activation. Proteases are generated and released as a result of immune responses (e.g. from cytotoxic T lymphocytes, polymorphs and killer cells involved in immune reactions, as a result of target cell lysis, and following events such as ADCC and complement activation). In addition to this physiological liberation of proteases, tumour cells carry surface proteases which are released by shedding, and also, proteases are released following cell death. However, in contrast to the physiological release of protease enzymes, proteases from tumour cells are produced unceasingly and in abundance. A mechanism is required to limit the activity of physiologically released protease to regulate the protease-dependent aspects of the immune response, and such a mechanism is provided by naturally occurring plasma protease inhibitors (PPI), of which A2M is the most important. The existence of PPI can be regarded as a means of

feedback control on cells capable of releasing proteases, as not only does A2M combine with and eliminate proteases rapidly but the A2M-P complex has inhibitory effects on the immune system. These actions include inhibition of lymphokine-induced cellular activity by lymphocyte recognition of antigen, inhibition of B cell activity, inhibition of the chemotactic activity of lymphokines and enzymes such as kallikrein and plasminogen activator, and regulation of lymphokine dependent activities of macrophages. The consequences of A2M combining with protease have been reviewed (Section 1.5.8), and it has been postulated that the SA of A2M-P complex is a result of a conformational change in the molecule[208]. Therefore, a tumour, which stimulates immune responses involving many cellular elements and which constantly sheds proteases from cell surfaces, would lead to a high level of complexed A2M. If the hypothesis proposed by Hubbard[208] is correct, then the A2M should be highly suppressive. The present study has demonstrated that the A2M found in cancer patients is highly suppressive with respect to lymphocyte reactivity, and confirms the hypothesis. This finding is of great significance because it demonstrates that, as a result of fulfilling its physiological function of inhibiting protease enzymes, the A2M molecule is able to suppress the activity of the immune system to a pathological degree. The importance of this role is emphasised by the present study which demonstrated clearly that A2M was the strongest plasma inhibitor of lymphocyte reactivity in all of the subjects examined.

It is known that the A2M-P complex is removed by cells of the reticulo-endothelial system following exposure of the receptor recognition site on the A2M molecule. The interaction of A2M-P and macrophages is therefore important from the aspect of removing A2M-P from the circulation,

but it may also be an important mechanism of immune cell suppression. Macrophages are essential for antigen presentation in immune recognition and may suppress or augment the immune response of lymphocytes (Section 1.2). Thus, it is possible that macrophages are a second target site for A2M-P, in addition to lymphocytes as demonstrated in this study. It is also attractive to link two apparent functions of macrophages - clearance of A2M-P and initiation of immune suppression as a result of interaction with A2M-P. Such a mechanism would act as its own inhibitory feedback loop by reducing immune reactivity and hence protease release from immune cells. However, with an additional source of proteases (e.g. release from a tumour) the feedback loop would be 'overloaded' and excessive or pathological immune inhibition would occur, as demonstrated by the present work.

Of particular interest to the present work are the studies by Remold et al[25,26] who examined the effect of plasma enzyme inhibitors, including A2M, on the responses of guinea pig macrophages to migration inhibition factor (MIF), a lymphokine which inhibits the migratory movement of macrophages. Remold found that the plasma esterase inhibitors A2M, alpha-1-antitrypsin, C1-inhibitor, antithrombin-heparin co-factor and soybean trypsin inhibitor enhanced responses of guinea pig macrophages to the action of MIF at physiological concentrations when cells were pre-incubated with the inhibitors before addition of MIF; the inhibitors themselves did not alter macrophage migration. Remold[26] concluded that the presence of serine esterases on the macrophage surface indicated that either they were inactivators of MIF or they participated in enzymatic interactions which resulted in activation of macrophages. Thus, it would appear that protease inhibitors, such as A2M, have an <u>in vitro</u> function in the regulation of macrophage activities by a complex interaction with

lymphokines and their inhibitors. If, for example, protease inhibitors (A2M) came into contact with macrophages at an inflammatory site or site at which an immunological response occurred then the macrophage response to lymphokines could be increased and the functional response of macrophages heightened. Remold's studies[25,26] indicate that A2M and similar plasma protease inhibitors have an influence on macrophages via lymphokine activity. The failure of A2M to influence macrophage migration could be because A2M does not have such a physiological role, or because the A2M used in the experiments was in the electrophoretically slow or non-complexed form.

A2M may influence other immune cells in addition to lymphocytes and macrophages. NK cell activity is impaired following interaction with proteases and so A2M would be expected to benefit NK activity. However, it is unknown whether the affinity of proteases for A2M is greater than for protease - sensitive sites on NK cells, and whether NK activity can be restored by PPI. Similarly, the effect of complexed A2M-P (as opposed to free A2M) on NK cells is not known, but the effect may be one of inhibition if the postulate of a local negative feedback mechanism is correct.

It is clear, then, that the biological behaviour of A2M may vary, depending on production of proteases. The A2M-P complexes are removed rapidly from plasma by reticulo-endothelial cells and hepatocytes (Section 1.5.8), although A2M levels in plasma were not significantly different in cancer patients and control subjects. Thus, A2M must be replenished as rapidly as it is removed, indicating that in patients suffering from conditions associated with the presence of the immunosuppressive form of A2M (A2M-P, the electrophoretically fast form of A2M), the turnover of A2M must be increased rapidly. This postulate is

supported by studies of patients with minor burns, showing significantly increased A2M turnover but normal serum concentrations[199]. If formation and removal of A2M-P complexes occurs at a rate faster than the provision of non-complexed A2M, then eventually all available A2M will be in the form of A2M-P and a maximal level of inhibitory activity due to A2M will be achieved. As A2M levels remain approximately constant in all individuals, the removal of A2M-P must in someway provide a stimulus to the formation of uncomplexed A2M and increase A2M turnover.

6.5.2 SMALL MOLECULAR WEIGHT PEPTIDE FRACTION

It has been proposed that combination of A2M with proteases causes or requires cleavage of a small MW peptide from the A2M molecule[183,184]. The function of this peptide is unknown, although Hubbard[208] postulated that it could act as a messenger rather than being expended after breaking from the parent molecule. The most obvious function would be related to control of protease production, i.e. the peptide could have a role in feedback inhibition of immune responses, which may have led to protease release initially. Such an action would increase the immunosuppressive effect of the A2M-P interaction which generates not only the electrophoretically fast form of A2M but an immunosuppressive peptide. The present study has not confirmed this propostion directly but has provided supportive evidence consistent with the postulate. Region 6 had a MW range of less than 50,000 daltons, and had considerable SA against autologous and healthy allogeneic lymphocytes when the region was derived from patients with cancer or who were malnourished. As discussed above, cancer plasma contained highly suppressive A2M (A2M complexed with proteases), and the detection of a small MW peptide fraction with SA is consistent with the postulates of Barrett et al[183,184] and Hubbard[208], and could have been

predicted from the results obtained from studying region 3 (Section 3.8). The present study has demonstrated an association between an A2M moiety with strong inhibitory activity and a small MW peptide region with lymphocyte inhibitory activity. Further studies performed in our laboratory have shown that this peptide fraction liberated by A2M following interaction with protease does not affect NK cell activity against K562 erythroleukaemic cells or ADCC against Chang liver cell targets[383].

6.5.3 IMMUNE COMPLEXES

Immune complexes (IC) were detected only in plasma from cancer patients and had SA against autologous and healthy allogeneic lymphocytes. Further studies of the specificity of antibodies and the nature of antigens comprising these IC were beyond the scope of this thesis, but, on the basis of the evidence reviewed (Section 1.5.7(a)), it is reasonable to assume that IC were formed from anti-tumour antibodies and tumour antigens. The finding that IC had lymphocyte SA provides further evidence that tumour antigens and their specific antibodies may be beneficial to a tumour by causing lymphocyte suppression. Antigen shedding (Section 1.5.3(b)) helps malignant cells evade immune destruction by preventing attachment of immune cells and antibodies to surfaces of malignant cells, by producing high concentrations of antigen locally and thereby "mopping up" anti-tumour antibody, by reducing tumour cell immunogenicity, and in some cases by their own inherent immunosuppressive activity. Antibodies may aid tumours by masking antigenic sites from immune detection and encouraging antigenic modulation. The present study has identified a further mechanism: antigen shedding and combination with antibody leads to the formation of complexes which can circulate freely and inhibit the reactivity of lymphocytes. The

amount of SA associated with IC was relatively small but nevertheless it contributed to reduce lymphocyte responsiveness.

IC were detected in all cancer patients analysed in detail in this study. Others[145] have demonstrated IC in the majority of cancer patients. Thus, it appears a characteristic of solid tumours that they shed surface antigens, which combine with host factors hostile to them to produce substances which reduce the efficiency of the host's anti-tumour response. The formation of IC can be seen as a mechanism which aids the immunological escape of tumours.

It is now clear that NK cells have a complex immune function in addition to their cytotoxic activity. An NK cell subset has been shown to inhibit B cell production of immunoglobulin, an effect which can be abrogated by complement-mediated lysis of NK cells[407]. Modulation of the Fc receptor of NK cells by IC greatly increases inhibition of the antibody response while at the same time decreasing cytotoxicity against standard targets of NK cell activity[408]. Hence, IC are able to reduce the activity of cells with inherent cytotoxicity against cancer cells, a property which may be of direct benefit to cancer cells.

6.5.4 Fc FRAGMENTS

Fc fragments of IgG in the low MW fraction of plasma from cancer patients have been shown to possess non-specific lymphocyte inhibitory activity. The specificity of the immunoglobulin from which the Fc fragments were derived is not known, but the reviewed evidence (Sections 1.4.2 and 1.5.7) indicates that IgG-Fc could have been derived from anti-tumour antibodies as a result of the action of tumour surface-associated protease enzymes[142]. Splitting tumour-associated IgG in this way would leave the Fab portion of the molecule attached to and obscuring

antigenic determinants on the tumour cell surface and render ineffective any method of tumour cell destruction which relied on an intact antibody molecule, such as ADCC and CDL. In addition, free Fc fragments could attach to Fc receptors on the surface of tumour cells and could possibly interfere with binding of anti-tumour antibodies.

Lymphocytes, macrophages, polymorphonuclear leukocytes and NK cells have surface receptors for Fc. Their precise role in some instances is uncertain, but it is clear that blockade of these surface receptors can interfere with cellular functions[87]. This has been confirmed by the present study which has shown that Fc fragments impair lymphocyte reactivity to antigenic and mitogenic stimuli. Fc fragments could inhibit or regulate other immune activities as well, for example, the Fc moiety of IC is responsible for inactivation of antigen-sensitive B lymphocytes[407].

Thus, detection of Fc fragments with non-specific SA can be interpreted as a further mechanism of immunological escape. Not only is the tumour cell able to cleave "anti-tumour" immunoglobulin but one of the cleavage products confers advantage on the tumour cell by reducing the function of the key cell in the immune system.

6.6 HYPOTHESES

The experimental work has confirmed the hypotheses stated in Section 1.8. The first hypothesis, that malignant disease is associated with non-specific immunosuppressive factors, has been confirmed by demonstrating that plasma from cancer patients inhibits the reactivity of autologous and healthy allogeneic lymphocytes to antigenic and mitogenic stimulation. The animal experiments showed that PSA increased with tumour growth and decreased following tumour removal, indicating that PSA was

related directly to the presence of tumour tissue. The central role of A2M in immune regulation has been demonstrated by detailed analysis of plasma samples. The ability of A2M to change its biological behaviour has been inferred by comparison of the immunosuppressive activity of A2M in the various subject groups; studies of A2M and in vitro combination with protease, and of the immunosuppressive ability of the A2M-P complex, will further support the hypothesis. The non-specific nature of the role of A2M has been highlighted by finding that A2M had SA in all subjects and that it was highly suppressive in all cancer patients irrespective of site or stage of their primary disease.

The study has shown that malnutrition in hospitalised patients with benign or malignant disease is associated with increased PSA, which is due largely to the increased immunosuppressive activity of A2M, and has confirmed the second hypothesis. In addition, a new functional method of immunological assessment of malnourished subjects with benign disease has been defined by the demonstration of a strong, statistically significant correlation between PSA and conventional methods of nutritional assessement. The third hypothesis, that TPN fluids containing fat emulsions will affect immune function adversely, has been proven by examining lymphocyte reactivity after in vivo and in vitro addition of fat-free and fat-containing intravenous fluids. The clinical implications of these observations cannot be ascertained from the present work.

Six experimental aims were given in Section 1.8. The experiments have fulfilled these aims, although the fifth objective, namely assessment of the relationship between SA of plasma and tumour growth in an animal model has not been investigated thoroughly. This is due largely to the nature of the animal model and the use of blood transfusion to induce high PSA (Section 5.5). The experiments reported in Chapter 5 are of great

interest, but strictly speaking, were not able to provide an answer as to the value of high PSA to tumour growth. Better design of the experiment could have provided data of greater relevance, although if the blood transfusion model had not been used, a fascinating area of tumour research might not have become apparent (viz., the influence of blood transfusion on tumour growth).

6.7 CONCLUSION

A2M, IC, Ig - Fc and small MW fragments have lymphocyte SA in cancer patients. These substances or the parent molecules are produced by the interaction of the host with a tumour. Some are released from tumour cell surfaces (proteases, antigens, immunoglobulins), and so, logically, local concentrations of complexed A2M (A2M-P), IC, Fc fragments and peptides would be greater than concentrations in peripheral blood, and although the plasma of cancer patients is suppressive, it is likely that the immediate micro-environment of a tumour is many times more suppressive. Such a situation would allow the tumour to proliferate in the presence of a specific immune response: circulating PB immune cells would still exhibit specific responses to tumour extracts and maintain their reactivity (although perhaps somewhat reduced), but those cells within the local environment of the tumour would be paralysed by the relatively high concentration of naturally occurring immunosuppressive factors. The concept is supported by the studies of Holmes[96] who demonstrated that the immune cells obtained from within tumours had a marked "functional paralysis" in comparison with PB cells in cancer patients. Thus, the study of naturally occurring immunosuppressive substances provides an explanation for the apparent paradox of tumour growth in the presence of specific anti-

tumour immune responses.

6.8 FUTURE STUDIES

The present study has set a foundation for future studies of suppressive factors in plasma. Few research projects produce complete answers in themselves but all should give direction to further work related to their subject.

Future studies should investigate the suppressive effects of plasma in other assays of immunological function - in particular, assays of B lymphocyte activity, other assays of lymphocyte activity (such as transformation), NK cell activity, ADCC and T lymphocyte cytotoxicity, as well as macrophage and polymorphonuclear leucocyte activity. Also, A2M should be obtained from other groups of patients with high and low SA and its activity measured in these assay systems. It has been suggested in this thesis that A2M turnover in patients with high PSA may be increased, and clearly this needs to be verified. It appears that the A2M-P complex is a highly immunosuppressive substance and this requires confirmation in other *in vitro* and *in vivo* systems.

Undoubtedly, identification of a naturally occurring immunosuppressive peptide in a number of diverse clinical conditions is an exciting observation. The range of activity of the peptide, its chemical composition and purification are being studied currently by others in the laboratory. The value of this peptide as an immunosuppressant must be investigated in animal models, with research being directed towards its possible use as a clinical immunosuppressive agent. Obviously, a considerable amount of work remains to be done before clinical use can be contemplated seriously, but even now the advantages of a naturally occurring immunosuppressive agent in terms of toxicity, activity and

availability are apparent. The other components of region 6, the small MW region of plasma, require definition as other immunosuppressive substances are present within this region and might be suitable for clinical or experimental purposes. It is conceivable that these naturally occurring substances could have a role in manipulating immune responses in circumstances in which they are required for short periods of time (e.g. exacerbations of auto-immune diseases, acute rejection of organ allografts or in the treatment of graft versus host disease following bone marrow transplantation).

It has been suggested that PSA could be used as an indicator of malignant disease in adequately nourished subjects. This proposal could be tested by measuring PSA in large populations of patients with malignant and non-malignant disorders. Large scale testing would require automation of the TEEM test technique, but is essential to determine the specificity and sensitivity of the test and its value in clinical practice.

The animal experiments indicate that PSA could be of prognostic value. The relevance of these studies to humans is unknown at present. A prospective study has been set up in the Department of Surgery, University of Newcastle-upon-Tyne, to measure PSA sequentially in patients before and after 'curative' surgery for malignant disease of the stomach, pancreas, colon, rectum and breast. Even after years of apparently good health following cancer surgery, some patients die of disseminated malignancy and any study assessing the value of a 'marker' of malignant disease must be extended over a long period of time before conclusions can be stated with confidence. However, such a study is essential to define the clinical significance of PSA, if any. Similarly, the use of PSA as a nutritional index requires further evaluation before it is of value in assessment of

malnutrition; it is essential to sequentially observe PSA in subjects receiving nutritional therapy, and to establish clinical correlates with changes in lymphocyte reactivity, PSA and A2M behaviour if they are to be accepted as contributing to patient management.

Perhaps the most important studies will be those involving the therapeutic manipulation of A2M in cancer patients. If the immunosuppressive effects of A2M and other naturally occurring substances can be decreased or the substances themselves reduced quantitatively, then it is likely that the immune system of cancer patients will be better able to detect and eliminate malignant tumours. Further work is needed to identify mechanisms to achieve this, especially within the micro-enviroment of tumours. Perhaps removal of proteases and other CN-SIF by processes akin to dialysis or plasmapheresis could be used in conjunction with conventional cytoreductive therapy. Alternatively, use of synthetic agents which compete with A2M for proteases would reduce the A2M-P load and reduce local and systemic tumour-associated immune depression.

APPENDICES

APPENDIX 1: CLINICAL STAGING OF THE MALIGNANT DISEASES

Clinical staging of breast cancer

Stage	T	N	M
1	1 , 2	0	0
2	1 , 2	1	0
3	1 - 4	2 , 3	0
4	1 - 4	0 - 3	1

Clinical staging of gastric, pancreatic, colonic and rectal cancers

Stage	T	N	M
1	1 , 2	0	0
2	3 , 4	0	0
3	1 - 4	1 - 3	0
4	1 - 4	1 - 3	1

APPENDIX 2: ALSEVERS MEDIUM

1. Dextrose 20.5 g/l
2. Sodium citrate 8.0 g/l
3. Sodium chloride 4.2 g/l

Dissolved in 1.0 litre of deionised water

APPENDIX 3: STAVITSKY'S PHOSPHATE BUFFERED SALINE (PBS)

1. KH_2PO_4 20.41 g/l (0.15 M)
2. Na_2HPO_4 21.29 g/l (0.15 M)

Add 119.5 ml 0.15 M KH_2PO_4

380 ml 0.15 M Na_2HPO_4

29.2 g NaCl

500 ml deionised water

Adjusted to pH 7.2

APPENDIX 4: HANK'S BALANCED SALT SOLUTION (BSS)

1.	Na_2HPO_4	0.06 g
2.	$MgSO_4 \cdot 7H_2O$	0.2 g
3.	KCl	0.4 g
4.	NaCl	8.0 g
5.	$CaCl_2$	0.14 g
6.	KH_2PO_4	0.06 g
7.	$NaHCO_3$	0.35 g

Dissolved in 1.0 litre of deionised water

APPENDIX 5: TRIS-BUFFER USED FOR GEL FILTRATION COLUMN CHROMATOGRAPHY

1.	Tris-hydroxy-methyl-methylamine	12.114 g
2.	Sodium chloride	9.0 g
3.	Sodium azide	2.0 g

Dissolved in 1.0 litre of deionised water

Adjusted to pH 7.4

APPENDIX 6: DETAILS OF GEL FILTRATION CHROMATOGRAPHY

Gel: Ultrogel AcA 22 (LKB - Produkter AB, Sweden)
Column dimensions: 2.5 cm x 95 cm
Cross-sectional area: $\pi r^2 = 3.1415 \times (1.25)^2 = 4.90\ cm^2$
Gel height: 82 cm
Gel bed volume: 402.5 cm^2 (86% of column volume)
Buffer: Tris-hydroxy-methyl-methylamine, pH 7.4, 0.1 M
Packing flow rate: 17.0 cm^3 per hour
Fractionation flow rate: 15.0 cm^3 per hour
Void volume: 100 - 115 ml (the first 20 -23 fractions)
Fraction volume: 5 ml
Fractions collected: 100
Time for collection of one fraction: 20 minutes
Time for complete separation of plasma sample: 33 1/3 hours
Paper speed of chart recorder: 2 cm per hour

APPENDIX 7: TRIS-BARBITONE BUFFER (TBB) USED FOR IMMUNO-ELECTROPHORESIS STUDIES

1.	Tris-hydroxy-methyl-methylamine	44.3 g
2.	Sodium barbitone	22.4 g
3.	Sodium azide	2.0 g

Dissolved in 1.0 litre of deionised water to produce the stock solution

Adjusted to pH 8.6

Diluted for use with deionised water: buffer + deionised water = 1 + 4

APPENDIX 8: COOMASIE BRILLIANT BLUE SOLUTION

1.	Coomasie brilliant blue	5.0 g
2.	Ethanol	450 ml
3.	Glacial acetic acid	100 ml
4.	Deionised water	450 ml

APPENDIX 9: DESTRAINER

1.	Ethanol 96%	250 ml
2.	Glacial acetic acid	100 ml
3.	Deionised water	450 ml

APPENDIX 10: DILUTIONS OF STANDARD REFERENCE PROTEINS AND TEST SERA IN SINGLE ROCKET ELECTROPHORESIS EXPERIMENTS

Standard proteins

Protein	Concentration (mg/100 ml)	Dilutions				Dilutions of test sera	
IgM	97	1+0,	1+1,	1+3,	1+10	1+5,	1+10
IgG	1256	1+20,	1+30,	1+50,	1+100	1+25,	1+50
Alpha-2 macroglobulin	205	1+5,	1+10,	1+20,	1+50	1+10,	1+20
Albumin	5035	1+200,	1+300,	1+500,	1+1200	1+250,	1+500

APPENDIX 11: WALPOLES ACETATE BUFFER

1.	Acetic acid	12.0 g/l	(0.2 molar)
2.	Sodium acetate	16.4 g/l	(0.2 molar)

Add 44.0 ml of 0.2 M acetic acid
and 6.0 ml of 0.2 M sodium acetate

Adjusted to pH 4.0

APPENDIX 12: CLARK - LUBBS BORATE BUFFER

Solution A

1.	Boric acid	6.2 g/l	(0.1 molar)
2.	Potassium chloride	7.4 g/l	(0.1 molar)

Solution B

1.	Sodium hydroxide	4.0 g/l	(0.1 molar)

Add 50 ml solution A
and 8.5 ml solution B
and 41.5 ml deionised water

Adjusted to pH 8.0

APPENDIX 13: RESULTS OF LYMPHOCYTE REACTIVITY TO PPD OF EACH OF THE THREE LYMPHOCYTE DONORS

Volunteer	Lymphocyte Reactivity (% TSRBC slowing)	
	n	mean ± 1SD
A	30	19.07 ± 0.33
B	31	19.15 ± 0.60
C	52	19.24 ± 0.78

n = number of occasions on which estimations were made

APPENDIX 14: FINAL VOLUMES (ml) OF THE CONCENTRATED REGIONS

Subject	Region			
	1	3	4	6
Normal				
1	-	5.2	-	-
2	-	5.5	-	-
3	-	2.4	-	-
4	-	5.1	-	-
5	-	5.3	-	-
Benign well nourished				
1	-	4.8	-	-
2	-	6.2	-	-
3	-	2.6	-	-
4	-	3.9	-	-
5	-	5.0	-	-
Benign malnourished				
1	-	3.1	4.6	4.3
2	-	5.6	4.5	3.0
3	-	6.3	6.0	2.6
4	-	6.2	7.0	7.9
5	-	6.7	2.9	5.1
Malignant well nourished				
1	2.0	4.5	7.3	4.8
2	2.6	4.2	6.9	3.8
3	2.7	5.6	6.6	5.7
4	2.9	6.0	6.5	6.0
5	1.4	5.9	4.5	2.4
6	5.2	2.4	6.3	1.7
Malignant malnourished				
1	3.5	2.9	6.5	5.7
2	2.3	5.0	7.9	5.6
3	4.0	3.5	8.3	4.5
4	2.3	3.9	5.9	3.0
5	5.2	6.2	8.5	5.4
6	4.2	4.3	6.0	4.3

APPENDIX 15: RATE OF TRIGLYCERIDE INFUSION

Infusion rate = 667 ml of 20% Intralipid/day

= 667 ml of 20 g triglyceride/100 ml/day

= 667 ml of 200 mmol triglyceride/100 ml/day

= 133.4 mmol triglyceride/day

= 0.0926 mmol triglyceride/minute

Mean body weight of subjects in group 3 = 64.9 kg

Plasma volume = 43 ml/kg body weight

= 2.79 litres

Mean increase in plasma triglyceride

= 0.0926 mmol/min x 2.79 l^{-1}

= 0.033 mmol triglyceride/l/min

= 33.0 μmol triglyceride/l/min

REFERENCES

1. Burnet M. Cancer - a biological approach. Parts I and II. Brit. Med. J., 1957, 1: 779-786.

2. Burnet M. Cancer - a biological approach. Parts III and IV. Brit. Med. J., 1957, 1: 841-847.

3. Thomas L. Reactions to homologous tissue antigens and relation to hypersensitivity. In: Cellular and humoral aspects of the hypersensitive states. Ed: Lawrence HS. Harper, New York, 1959, 529-532.

4. Burnet FM. The concept of immune surveillance. Prog. Exp. Tumor Res., 1970, 13: 1-27.

5. Smith RT, Landy M. Immune surveillance - proceedings of an international conference. Academic Press, New York, 1970.

6. Ioachim HL. The stromal reaction of tumours: an expression of immune surveillance. J. Natl. Cancer Inst., 1976, 57: 465-475.

7. Moller G, Moller E. Considerations of some current concepts in cancer research. J. Natl. Cancer Inst., 1975, 55: 755-759.

8. Outzen HC, Custer RP, Eaton GJ, Prehn RT. Spontaneous and induced tumor incidence in germ free "nude" mice. J. Reticuloendothl. Soc., 1975, 17: 1-9.

9. Schwartz RS. Another look at immunologic surveillance. N. Eng. J. Med., 1975, 293: 181-184.

10. Currie G. Cancer and the immune response. Edward Arnold Publishers, London, 1980, 4-72.

11. Rosenberg EB, McCoy JL, Green SS, et al. Destruction of human lymphoid tissue-culture cell lines by human peripheral lymphocytes in ^{51}Cr release cellular cytotoxicity assays. J. Natl. Cancer Inst., 1974, 52: 345-352.

12. Calderon J, Kieily JM, Lefko JL, Uanue ER. The modulation of lymphocyte functions by molecules secreted by macrophages. I. Description and partial biochemical analysis. J. Exp. Med., 1975, 142: 151-164.

13. Rosenthal AS, Shevach EM. The function of macrophages in T-lymphocyte antigen recognition. In: Contemporary Topics in Immunobiology. Ed: Weigle WO. Plenum Press, New York, 1976, 5: 47-90.

14. Fundenberg HH, Stites DP, Caldwell JL, Wells JV. Basic and clinical immunology. 2nd Ed. Lange Medical Publications, California, 1978, p.160.

15. Greineder DK, Rosenthal AS. Macrophage activation of allogeneic lymphocyte proliferation in the guinea pig mixed leukocyte culture. J. Immunol., 1975, 114: 1541-1547.

16. Rosenthal AS, Shevach EM. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. J. Exp. Med., 1973, 138: 1194-1212.

17. Shevach EM, Paul WE, Green I. Histocompatibility-linked immune response gene function in guinea pigs. Specific inhibition of antigen induced lymphocyte proliferation by allosera. J. Exp. Med., 1972, 136: 1207-1221.

18. Binz H, Wegzell H. T cell receptors with allo-major histocompatiblity complex specificity from rat and mouse. J. Exp. Med., 1981, 154: 1261-1278.

19. Bretscher PA. The two signal model for B cell induction. Transplant Rev., 1975, 23: 37-48.

20. Bretscher PA. Hypothesis: On the control between cell mediated, IgM and IgG immunity. Cell. Immunol., 1974, 13: 171-195.

21. Dumonde DC, Wolstencroft RA, Panayi GS, Matthew M, Morely J, Howson WT. 'Lymphokines': non-antibody mediators of cellular immunity generated by lymphocyte activation. Nature, 1969, 224: 38-42.

22. Maini RN, Bryceson ADM, Wolstencroft RA, Dumonde DC. Lymphocyte mitogenic factor in man. Nature, 1969, 224: 43-44.

23. Rocklin RE, Bendtzen K, Greinder D. Mediators of immunity: lymphokines and monokines. Adv. Immunol., 1980, 29: 55-136.

24. Cohen S, Yoshida T. Suppression of B cell MIF production by T cells and soluble T cell derived factors. J. Immunol., 1977, 119: 719-721.

25. Remold HG. The enhancement of MIF activity by inhibition of macrophage associated esterases. J. Immunol., 1974, 112: 1571-1577.

26. Remold HG, Rosenberg RD. Enhancement of migration inhibition factor activity by plasma esterase inhibitors. J. Biol. Chem., 1975, 250: 6608-6613.

27. Sanderson CJ, Glauert AM. The mechanism of T cell mediated cytotoxicity. VI. T cell projections and their role in target cell killing. Immunology, 1979, 36: 119-129.

28. Berke G, Ax W, Ginsburg H, Feldman M. Graft reaction in tissue culture. II. Quantification of the lytic action on mouse fibroblasts by rat lymphocytes sensitized on mouse embryo monolayers. Immunology, 1969, 16: 643-657.

29. Doherty PC, Zinkernagel RM. T cell mediated immunopathology in viral infections. Transplant Rev., 1974, 19: 89-120.

30. Biozzi G, Stiffel C, Mounton D, Bouthillier Y. Selection of lines of mice with high and low antibody responses to complex immunogens. In: Immunogenetics and Immunodeficiency. Ed: Benacerraf B., MT Press, Lancaster, 1975, 179-227.

31. Benacerraf B, Katz DH. The histocompatibility immune response genes. Adv. Cancer Res., 1975, 21: 121-173.

32. Davis BK, Kunz HW, Shonnard JW, Gill TJ. Immune response to poly (Glu^{52} Lys^{33} Tyr^{15}) in congenic rats. Transplant Proc., 1981, 13: 1378-1382.

33. Basten A, Loblay RH, Trent RT, Gatenby PA. Suppressor T cells in immune homeostasis and disease. In: Recent Advances in Clinical Immunology. Ed: Thompson RA. vol 2. Churchill Livingstone, Edinburgh, 1980, 33-63.

34. Loblay RH, Pritchard-Driscoe H, Basten A. Suppressor T-cell memory. Nature, 1978, 272: 620-622.

35. Ramshaw IA, McKenzie IFC, Bretscher PA, Parish CR. Discrimination of suppressor T cells of humoral and cell-mediated immunity by anti-Ly and anti-Ia sera. Cellular Immunology, 1977, 31: 364-369.

36. Dorsch S, Roser B. Recirculating suppressor T cells in transplantation tolerance. J. Exp. Med., 1977, 145: 1144-1157.

37. Haynes BF, Fauci AS. Activation of human B lymphocytes. J. Immunol., 1977, 118: 2281-2287.

38. Mosier DE, Mathieson BJ, Campbell PS. Ly phenotype and mechanism of action of mouse neonatal suppressor T cells. J. Exp. Med., 1977,146: 56-73.

39. Roder JC, Duwe AK, Bell DA, Singhal SK. Immunological senescence. I. The role of suppressor cells. Immunology, 1978, 35: 837-847.

40. Cooke A, Hutching RR, Playfair JHL. Suppressor T cells in experimental autoimmune haemolytic anaemia. Nature, 1978, 273: 154-155.

41. Katz DR. Immunosuppression as a physiological homeostatic mechanism. Immunology Today, 1984, 5: 96-97.

42. Jerne NK. Towards a network theory of the immune system. Ann. Immunol., 1974, 125: C, 373.

43. Rogers MJ. Tumor-associated transplantation antigens of chemically-induced tumors: new complexities. Immunology Today, 1984, 5: 167-168.

44. Prehn RT. In: Cross-reacting antigens and neoantigens: with implications for auto-immunity and cancer immunity. Ed: Trentin J. Williams and Wilkins, Baltimore, 1967, (quoted by Currie [10]).

45. Ristow S, McKhann CF. Tumour associated antigens. In: Mechanisms of Tumour Immunity. Ed: Green I, Cohen S, McClusky RT. John Wiley & Sons, New York, 1977, 109-145.

46. Haywood GR, McKhann CF. Antigenic specificities on murine sarcoma cells. J. Exp. Med., 1971, 133: 1171-1187.

47. Edynak EM, Hirshant Y, Bernard M, Tempe G. Fluorescent antibody studies on human breast cancer. J. Nat. Cancer Inst., 1972, 48: 1137-1143.

48. Nairn RC, Nind APP, Guli EPG, Davies DJ, Rolland JM, McNiven AR, Hughes ESR. Immunological reactivity in patients with carcinoma of colon. Brit. Med. J., 1971, 4: 706-709.

49. Lewis MG, Phillips TM, Cook KB, Blake J. Possible explanation for loss of detectable antibody in patients with disseminated malignant melanoma. Nature, 1971, 232: 52-53.

50. Thunold S, Abeyounis CJ, Milgrom F, Witebsky E. Anti-gamma globulin factors in serum and tissue of cancer patients. Int. Arch. All. Appl. Immunol., 1970, 38: 260-268.

51. Humphrey LJ, Volenec PJ, Volenec FJ, Cross D. Ante-antibodies to an altered IgG in human breast cancer. J. Surg. Oncol., 1977, 9: 29-37.

52. Roberts MM, Bass EM, Wallace IWJ, Stevenson A. Local immunoglobulin production in breast cancer. Brit. J. Cancer, 1973, 27: 269-275.

53. Hurlimann J, Lichaa M, Ozzello L. In Vitro synthesis of immunoglobulins and other proteins by dysplastic and neoplastic human mammary tissues. Cancer Res., 1976, 36: 1284-1292.

54. Ran M, Klein G, Witz IP. Tumour-bound immunoglobulins. Evidence for the In Vitro coating of tumour cells by potentially cytotoxic anti-tumour antibodies. Int. J. Cancer, 1976, 17: 90-97.

55. Moller E. Contact-induced cytotoxicity by lymphoid cells containing foreign isoantigens. Science, 1965, 147: 873-879.

56. Basham C, Currie GA. Development of specific cell-dependent antibody during growth of a syngeneic rat sarcoma. Brit. J. Cancer, 1974, 29: 189-198.

57. Haskill JS, Hayry P, Radov LA. Systemic and local immunity in allograft and cancer rejection. In: Contemporary Topics in Immunology, Vol. 8. Eds: Warner NL, Cooper MD. Plenum Press, New York, 1978, 107-170.

58. Herberman RB. Existence of tumour immunity in man. In: Mechanisms of Tumour Immunity. Eds: Green I, Cohen S, McCluskey RT. John Wiley & Sons, New York, 1977, 175-191.

59. Francis DMA, Shenton BK. Depression of cellular immunity as an index of malnutrition in surgical patients. Brit. Med. J., 1981, 282: 1474.

60. Char DH, Lepourhiet A, Leventhal BG, Herberman RB. Cutaneous delayed hypersensitivity responses to tumor-associated and other antigens in acute leukemia. Int. J. Cancer, 1973, 12: 409-419.

61. Burton RC, Chism SE, Warner NL. In Vitro induction and expression of T cell immunity to tumor-associated antigens. In: Contemporary Topics in Immunobiology. Vol 8. Eds: Warner NL, Cooper MD. Plenum Press, New York, 1978, 69-106.

62. Bagshawe KD. Immunodiagnosis of cancer. In: Developments in Cancer Research. Vol 6. Eds: Preece AW, Light PA. Elsevier North Holland Biomedical Press, 1981, 17-23.

63. Cochran AJ. In Vitro immunological tests in the diagnosis of cancer. In: Developments in Cancer Research. Vol 6. Eds: Preece AW, Light PA. Elsevier North Holland Biomedical Press, 1981, 25-33.

64. Russel BRG. The nature of resistance to the inoculation of cancer. Third Scientific Report of the Imperial Cancer Research Foundation, 1908, 3: 341-358.

65. Ioachim HL, Dorsett B, Paluch E. The immune response at the tumour site in lung carcinoma. Cancer, 1976, 38: 2296-2309.

66. Carr I, Underwood JC. The ultrastructure of the local cellular reaction to neoplasia. Int. Rev. Cytology, 1974, 37: 329-347.

67. Ioachim HL. Correlations between tumour antigenicity, malignant potential and the local host immune response. In: Contemporary Topics in Immunology, Vol 10. Eds: Witz IP, Hanna MG. Plenum Press, New York, 1980, 213-238.

68. Lauder I, Aherne W. The significance of lymphocytic infiltration in neuroblastoma. Brit. J. Cancer, 1972, 26: 321-330.

69. Cochran AJ. Histology and prognosis in malignant melanoma. J. Pathol., 1968, 97: 459-468.

70. McGovern VJ, Mihm MC, Bailly C. The classification of malignant melanoma and its histologic reporting. Cancer, 1973, 32: 1446-1457.

71. Klein E, Becker S, Svedmyr E, Jondal M, Vanky F. Tumour infiltrating lymphocytes. Ann. N.Y. Acad. Sci., 1976, 276: 207-216.

72. Klein E, Vanky F, Galili U, Vose BM, Fopp M. Separation and characteristics of tumour-infiltrating lymphocytes in man. In: Contemporary Topics in Immunology, Vol 10. Eds: Witz IP, Hanna MG. Plenum Press, New York, 1980, 79-107.

73. Plata F, Sordat B. Murine sarcoma virus (MSV)-induced tumours in mice. I. Distribution of MSV immune cytolytic T lymphocytes in vivo. Int. J. Cancer, 1977, 19: 205-211.

74. Plata F, Gomard E, Lecler JC, Levy JP. Further evidence for the involvement of thymus-processed lymphocytes in syngeneic tumor cell cytolysis. J. Immunol., 1973, 111: 667-671.

75. Russell SQ, Gillespie GY, Hansen CB, Cochrane CG. Inflammatory cells in solid murine neoplasms. II. Cell types found throughout the course of Moloney sarcoma regression and progression. Int. J. Cancer, 1976, 18: 331-338.

76. Gillespie GY, Hansen CB, Hoskins RG, Russell SQ. Inflammatory cells in solid murine neoplasms. IV. Cytolytic T lymphocytes isolated from regressing or progressing Moloney sarcomas. J. Immunol., 1977, 119: 564-570.

77. James K, McBride B, Stuart A. The macrophage and cancer. Econoprint, Edinburgh, 1977.

78. Eccles SA, Alexander P. Macrophage content of tumours in relation to metastatic spread and host immune reaction. Nature, 1974, 250: 667-669.

79. Alexander P, Eccles SA, Gaucci CLL. The significance of macrophages in human and experimental tumours. Ann. N.Y. Acad. Sci., 1976, 276: 124-133.

80. Castro JE. Phagocytic cells and cancer. Brit. Med. J., Suppl. Health Care World Wide, 1976, 15: 1-4.

81. Kaplan AM, Morahan PS. Macrophage mediated tumour cell cytotoxicity. Ann. N.Y. Acad. Sci., 1976, 276: 134-145.

82. Hibbs JB, Lambert LH, Remington JS. Resistance to immune tumors conferred by chronic infection with intracellular protozoa, toxoplasma gondii and Besnoitia jellisoni.

83. Evans R, Alexander P. Co-operation of immune lymphoid cells with macrophages in tumour immunity. Nature, 1970, 228: 620-622.

84. Piessens WF, Churchill WH, David JR. Macrophages activated in vitro with lymphocyte mediators kill neoplastic but not normal cells. J. Immunol., 1975, 114: 293-299.

85. Granger GA, Weiser RS. Homograft target cells: contact destruction in vitro by immune macrophages. Science, 1966, 151: 97-99.

86. Herberman RB, Holden HT. Natural cell-mediated immunity. Adv. Cancer Research, 1978, 27: 305-377.

87. Peter HH, Kalden JR, Seeland P, Diehl V, Eckert G. Humoral and cellular immune reactions in vitro against allogeneic and autologous human melanoma cells. Clin. Exp. Immunol., 1975, 20: 193-207.

88. Keissling R, Petranyi G, Karre K, Jondal M, Tracey D, Wigzell H. Killer cells: a functional comparison between natural, immune T cell and antibody -dependent in vitro systems. J. Exp. Med., 1976, 143: 772-785.

89. Singh G, Das SN, Khanna NN. Immune status in advanced upper gastro -intestinal cancers. J. Surg. Oncol., 1985, 29: 43-45.

90. Das SN, Khanna NN, Khanna S. A multiparametric observation of immune competence in breast cancer and its correlations with tumour load and prognosis. Ann. Acad. Med., 1985, 14: 374-381.

91. Haffejee AA, Webster DJJ, Paterson AG et al. E-rosette inhibition and tumour recurrence in early breast cancer. Br. J. Surg., 1983, 70: 319-321.

92. Anthony HM, Kirk JA, Madsen KE, Mason MK, Temple MAN. E and EAC rosetting lymphocytes in patients with carcinoma of the bronchus. Clin. Exp. Immunol., 1975, 20: 41-54.

93. Cochran AJ, Mackie RM, Grant RM et al. An examination of the immunology of cancer patients. Int. J. Cancer, 1976, 18: 298-309.

94. Lichtenstein A, Zighelboim J, Dorey F, Brossman S, Fahey JL. Comparison of immune derangements in patients with different malignancies. Cancer, 1980, 45: 2090-2095.

95 Kamo I, Friedman H. Immunosuppression and the role of suppressive factors in cancer. Adv. Cancer Res., 1977, 25: 271-321.

96. Holmes EC. Immunology of tumor infiltrating lymphocytes. Ann. Surg., 1985, 201:158-163.

97. Lilly F. Mouse leukaemia. A model of a multiple gene disease. J. Natl. Cancer Inst., 1972, 49: 927-934.

98. Kolsch E. The genetic control of T cell-mediated immunity against the DBA/2 mastocytoma P815. Eur. J. Immunol., 1975, 5: 527-532.

99. Martinez A. Radical irradiation without a mastectomy: an alternative to radical surgery in the treatment of early breast cancer. In: Recent Advances in Clinical Oncology, Vol 7. Ed: Williams CJ, Whitehouse JMA. Churchill Livingstone, Edinburgh, 1982, 354-362.

100. Kellerman G, Shaw CR, Luyten-Kellermann M. Amyl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. N. Eng. J. Med., 1973, 289: 934-937.

101. Cochran AJ. Histology and prognosis in malignant melanoma. J. Pathol., 1968, 97: 459-468.

102. Ioachim HL, Berwick L, Furth J. Replication of gross leukaemia virus in long term cultures of rat thymomas. Bio-assays and electron microscopy. Cancer Res., 1966, 20: 803-811.

103. Ioachim HL, Sabbath M. Redistribution and modulation of gross murine leukaemia virus antigens induced by specific antibodies. J. Natl. Cancer Inst., 1979, 62: 169-180.

104. Ioachim HL, Pearse A, Keller S. Antigenic deletion and malignant enhancement induced in lymphoma cells by passage through X irradiated hosts. Nature, 1977, 265: 55-57.

105. Ioachim HL, Dorsett B, Sabbath M, Keller S. Loss and recovery of phenotypic expression of gross leukaemic virus. Nature (New Biol.), 1972, 237: 215-218.

106. Goldman LI, Flazman BA, Wernick G, Zabriskie JB. Immune surveillance and tumor dissemination in vitro: comparison of the B16 melanoma in primary and metastatic form. Surgery, 1974, 76: 50-56.

107. Black MM, Zachram RE, Shore B, Leis HP. Biological considerations of tumour-specific and virus associated antigens of human breast cancers. Cancer Res., 1976, 36: 769-774.

108. Black PH. Shedding from the cell surface of normal and cancer cells. Adv. Cancer Res., 1980, 32: 75-199.

109. Murgita RA, Tomasi TB. Suppression of the immune response by alpha-fetoprotein. J. Exp. Med., 1975, 141: 269-286.

110. Murgita RA, Tomasi TB. Suppression of the immune response by alpha-fetoprotein. J. Exp. Med., 1975, 141: 440-452.

111. Koono M, Ushijima K, Hayashi H. Studies on the mechanisms of invasion in cancer. Int. J. Cancer, 1974, 13: 105-115.

112. Sylven B, Bois-Svensson I. On the chemical pathology of interstitial fluid. I. Proteolytic activities in transplanted mouse tumors. Cancer Res., 1965, 25: 458-468.

113. Wu R. Leakage of enzymes from ascites tumour cells. Cancer Res., 1959, 19: 1217-1222.

114. Holmberg B. On the in vitro release of cytoplasmic enzymes from ascites tumor cells as compared with strain L cells. Cancer Res., 1961, 21: 1386-1393.

115. Yefenof E. Lundin L, Klein G. Shedding of anti-IgM from antibody coated human lymphoma lines: differences between Epstein-Barr virus-negative lines and their virus-converted sublines. Eur. J. Immunol., 1978, 8: 190-193.

116. Burger MM. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. Nature, 1970, 227: 170-171.

117. Vischer TL. Stimulation of mouse B lymphocytes by trypsin. J. Immunol., 1974, 113: 58-62.

118. Hart DA, Streilein JS. Stimulation of a subpopulation of hamster lymphoid cells by trypsin and chymotrypsin. Exp. Cell. Res., 1976, 102: 246-252.

119. Gisler RH, Vischer TL, Dukor P. Trypsin increases in vitro antibody synthesis and substitutes for T helper cells. J. Immunol., 1976, 116: 1354-1357.

120. Bullock WW, Moller E. Spontaneous B cell activation due to loss of normal mouse serum suppressor. Eur. J. Immunol., 1972, 2: 514-517.

121. Lightman MA, Weed RI. Electrophoretic mobility and N acetyl neuraminic acid content of human normal and leukemic lymphocytes and granulocytes. Blood, 1970, 35: 12-22.

122. Geiger C, Vischer TC. Migration inhibition factor-like activity in inflammatory synovial fluids might be due to proteases. Clin. Exp. Immunol., 1976, 26: 176-180.

123. Hart DA, Streilein JS. Effect of protease inhibitors on mitogen stimulation of hamster lymphoid cells. Exp. Cell. Res., 1976, 102: 253-263.

124. Chen LB, Buchanan JM. Mitogenic activity of blood components. I. thrombin and prothrombin. Proc. Natl. Acad. Sci. U.S.A., 1975, 72: 131-135.

125. Chen LB, Teng NNH, Buchanan JM. Mitogenicity of thrombin and surface alterations on mouse splenocytes. Exp. Cell. Res., 1976, 101: 41-46.

126. Currie GA, Basham C. Serum mediated inhibition of the immunological reactions of the patient to his own tumour: a possible role for circulating antigen. Brit. J. Cancer, 1972, 26: 427-438.

127. Baldwin RW, Embleton MJ, Robins RA. Cellular and humoral immunity to rat hepatoma-specific antigens correlated with tumour status. Int. J. Cancer, 1973, 11: 1-10.

128. Baldwin RW, Embleton MJ, Price MR. Inhibition of lymphocyte cytotoxicity for human colon carcinoma by treatment with solubilised tumour membrane fractions. Int. J. Cancer, 1973, 12: 84-92.

129. Treves AJ, Carnaud C, Trainin N, Feldman M, Cohen IR. Enhancing T lymphocytes from tumor-bearing mice suppress host resistance to a syngeneic tumor. Eur. J. Immunol., 1974, 4: 722-727.

130. Fisher MS, Kripke ML. Further studies on the tumour specific suppressor cells induced by ultraviolet radiation. J. Immunol., 1978, 121: 1139-1144.

131. Fujimoto S, Greene MI, Sehon AH. Regulation of the immune response to tumour antigens. J. Immunol., 1976, 116: 800-806.

132. Greene MI, Dorf ME, Pierres M, Benacerraf B. Reduction of syngeneic tumour growth by an anti I-J allo antiserum. Proc. Nat. Acad. Sci. (U.S.A.), 1977, 74: 5118-5121.

133. Perry LL, Benacerraf B, Greene MI. Regulation of the immune response to tumour antigen. J. Immunol., 1978, 121: 2144-2147.

134. Zembala M, Mytar B, Popiela T, Asherson GL. Depressed in vitro peripheral blood lymphocyte response to mitogens in cancer patients: the role of suppressor cells. Int. J. Cancer, 1977, 19: 605-613.

135. Yu A, Watts H, Jaffe N, Parkman R. Concomitant presence of tumor-specific cytotoxic and inhibitor lymphocytes in patients with osteogenic sarcoma. New Eng. J. Med., 1977, 297: 121-127.

136. Izsak FC, Brenner HJ, Landes E, Ran M, Witz IP. Correlation between clinicopathological features of malignant tumours and cell surface immunoglobulins. Isr.J. Med. Sci., 1976, 10: 642-646.

137. Ting CC, Herberman RB. Specific afferent interference by antiserum of in vitro immunity. Nature, 1975, 257: 801-802.

138. Vanky F, Trempe G, Klein E, Stjernsward J. Human tumor-lymphocyte interaction in vitro: blastogenesis correlated to detectable immunoglobulins in the biopsy. Int. J. Cancer, 1975, 16: 113-124.

139. Yefenof E, Witz IP, Klein E. Interaction of antibody and cell surface localised antigen. Int. J. Cancer, 1976, 17: 633-639.

140. Witz IP. Tumour bound immunoglobulins:in situ expressions of humoral immunity. Adv. Cancer Res., 1977, 25: 95-148.

141. Ran M, Fish F, Witz IP, Klein G. Tumour-bound immunoglobulins. Clin. Exp. Immunol., 1974, 16: 335-353.

142. Fish F, Witz P, Klein G. Tumour-bound immunoglobulins. Clin. Exp. Immunol., 1974, 16: 355-365.

143. Cooper NR, Polley MJ, Oldstone MBA. Failure of terminal complement components to induce lysis of Moloney Virus transformed lymphocytes. J. Immunol., 1974, 112: 866-868.

144. Osther K. CI inactivator from cancer cells. Lancet, 1974, 1: 359-360.

145. Rossen RD, Reisberg MA, Hersh EM, Gutterman JU. The Clq binding test for soluble immune complexes. Clinical correlations obtained in patients with cancer. J. Natl. Cancer Inst., 1977, 58: 1205-1215.

146. Thomson DMP, Eccles S, Alexander P. Antibodies and soluble tumour-specific antigens in blood and lymph of rats with chemically induced sarcomata. Br. J. Cancer, 1973, 28: 6-15.

147. Thomson DMP, Sellens V, Eccles S, Alexander P. Radioimmunoassay of tumour specific transplantation antigen of a chemically induced rat sarcoma: circulating soluble tumour antigen in tumour bearers. Br. J. Cancer, 1973, 28: 377-388.

148. Thomson DMP, Steele K, Alexander P. The presence of tumour specific membrane antigen in the serum of rats with chemically induced sarcomata. Br. J. Cancer, 1973, 27: 27-34.

149. Sjogren HO, Hellstrom I, Bansal SC, Warner GA, Hellstrom KE. Elution of "blocking factors" from human tumors, capable of abrogating tumor-cell destruction by specifically immune lymphocytes. Int. J. Cancer, 1972, 9: 274-283.

150. Tonder O, Humphrey LJ, Morse PA. Further observations on Fc receptors in human malignant tissue and normal lymphoid tissue. Cancer, 1975, 35: 580-587.

151. Sinclair N. Fc receptors and Fc-mediated regulation. In: Strategies of immune regulation. Ed: Sercarz E, Cunningham AJ. Academic Press Inc. New York, 1980, 387-388.

152. Goodwin JS, Bankhurst AD, Messner RP. Suppression of human T cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. J. Exp. Med., 1977, 146: 1719-1734.

153. Gordon D, Bray MA, Morley J. Control of lymphokine secretion by prostaglandins. Nature, 1976, 262, 401-402.

154. Roder JC, Klein M. Target-effector interaction in the natural killer cell system. J. Immunol., 1979, 123: 2785-2790.

155. Zimecki M, Webb DR. The regulation of the immune response to T-independent antigens by prostaglandins and B cells. J. Immunol., 1976, 117: 2158-2164.

156. Sykes JAC, Maddox IS. Prostaglandin production by experimental tumours and effects of anti-inflammatory compounds. Nature (New Biol.), 1972, 237: 59-61.

157. Williams ED, Karim CMM, Sandler M. Prostaglandin secretion by medullary carcinoma of the thyroid. Lancet, 1968, 1: 22-23.

158. Bhana D, Hillier K, Karim SM. Vasoactive substances in Kaposi's sarcoma. Cancer, 1971, 27: 233-237.

159. Plescia OJ, Smith AH, Grinwich K. Subversion of immune system by tumour cells and role of prostaglandins. Proc. Natl. Acad. Sci. U.S.A., 1975, 72: 1848-1851.

160. Goodwin JS, Messner RP, Bankhurst AD, Peake GT, Saiki JH, Williams RC. Prostaglandin-producing suppressor cells in Hodgkin's disease. N. Engl. J. Med., 1977, 297: 963-968.

161. Smith RT, Landy M. Immunobiology of the tumor-host relationship. Academic Press, New York, 1975, p201-276.

162. Holmberg B. Further biochemical studies on a dialysis polypeptide obtained from tumor fluids. Eur. J. Cancer, 1968, 4: 263-269.

163. Glasgow AH, Nimberg RB, Menzoian JO et al. Association of anergy with an immunosuppressive peptide fraction in the serum of patients with cancer. N. Eng. J. Med., 1974, 291: 1263-1267.

164. Friedman H, Specter S, Watanabe M, Pan SH. Tumor-induced immunosuppression. Am. J. Pathol., 1978, 93: 499-514.

165. Kappler JW, Marrack P. Soluble effectors, mediators and factors and their mechanisms of action. In: Strategies of Immune Regulation. Eds: Sercarz E, Cunningham AJ. Academic Press, New York, 1980, p379-382.

166. Steinberg AD. The balance between helper and suppressor factors. In: Strategies of Immune Regulation. Eds: Sercarz E, Cunningham AJ. Academic Press, New York, 1980, p377.

167. Bankhurst AD. The role of non-neoplastic suppressor cells in the immunodeficiency associated with cancer. In: Suppressor cells in human disease. Ed: Goodwin JS. Marcel Dekker Inc., New York, 1981, p315-357.

168. Thomas DW, Roberts WK, Talmage DW. Regulation of the immune response production of a soluble suppressor by immune spleen cells in vitro. J. Immunol., 1975, 114: 1616-1622.

169. Tada T, Taniguchi M, Takemori T. Properties of primed suppressor T cells and their products. Transplant Rev., 1975, 26: 106-129.

170. Hanicki Z, Cichoki T, Sarnecka-Keller M, Klein A, Komorowska Z. Influence of middle-sized molecule aggregates from dialysis of uremic patients on lymphocyte transformation in vitro. Nephron, 1976, 17: 73-80.

171. Navarro J, Touraine J-L, Corre C, Traeger J. Prolongation of skin allograft survival and inhibition of graft versus host reaction in rodents treated with "middle molecules". Cell. Immunol., 1977, 31: 349-354.

172. Mabuchi H, Nakahashi H. Medium-sized peptides in the blood of patients with uremia. Nephron, 1983, 33: 232-237.

173. Fisher CL, Gill LW. Serum protein abnormalities. Little, Brown Pub., Boston, 1975.

174. Hiramatsu S, Kojima J, Okada TT, Inai S, Ohmori K. The serum protein profile in chronic hepatitis, cirrhosis and liver cancer. Acta Hepato-Gastroenteral., 1976, 23: 177-182.

175. Cooper EH, Stone J. Acute phase reactant proteins in cancer. Adv. Cancer Res., 1979, 30: 1-44.

176. Mortensen RF, Osmand AP, Gewurz H. Effects of C-reactive protein on the lymphoid system. J. Exp. Med., 1975, 141: 821-839.

177. Chiu KM, Mortensen RF, Osmand AP, Gewurz H. Interactions of alpha-1-acid glycoprotein with the immune system. Immunology, 1977, 32: 997-1005.

178. Menzoian JO, Glasgow AH, Nimberg RD et al. Regulation of T lymphocyte function by immuno-regulatory alpha-globulin. J. Immunol., 1974, 113: 266-273.

179. James K. A study of the alpha 2 macroglobulin homologues of various species. Immunology, 1965, 8: 55-61.

180. James K. Alpha 2 macroglobulin and its possible importance in immune systems. TIBS, 1980, 5: 43-47.

181. Harpel PC. Human alpha 2 macroglobulin. Methods Enzymol., 1976, 45: 639-652.

182. Laurell CB, Jeppsson JO. Protease inhibitors in plasma. In: The Plasma Proteins. Ed: Putnam FW. 2nd Edn. Vol. 1. Academic Press, New York, 1975, 229-264.

183. Starkey PM, Barrett AJ. In: Proteinases in Mammalian Cells and Tissues. Ed: Barrett AJ. North Holland, Amsterdam, 1977, 663-696.

184. Barrett AJ, Brown MA, Sayers CA. The electrophoretically "slow" forms of the alpha 2 macroglobulin molecule. Biochem. J., 1979, 181: 401-418.

185. Feinman RD (Ed). Chemistry and biology of a-2-macroglobulin. Ann. N.Y. Acad. Sci., 1983, 421: 1-478.

186. Feldman SR, Gonias SI, Pizzo SV. Model of alpha-2-macroglobulin structure and function. Proc. Natl. Acad. Sci. U.S.A., 1985, 82: 5700-5704.

187. Sottrup-Jensen L, Lonblad PB, Stepanik TM, Petersen TE, Magnusson S, Jornvall H. Primary structure of the "bait" region for proteinases in alpha-2 macroglobulin. FEBS. Letters, 1981, 167-173.

188. Imber MJ, Pizzo SV. Clearance and binding of two electrophoretic "fast" forms of human alph-2-macroglobulin. J. Biol. Chem., 1981, 256: 8134-8139.

189. Feldman SR, Rosenberg MR, Ney KA, Michalopoulos G, Pizzo SV. Binding of alpha-2-macroglobulin to hepatocytes: mechanism of in vivo clearance. Biochem. Biophys. Res. Com., 1985, 128: 795-802.

190. Ohlsson H. Elimination of ^{125}I-Trypsin alpha macroglobulin complexes from blood by reticulo-endothelial cells in dog. Acta Physiol. Scand., 1971, 81: 269-272.

191. Tunstall AM, James K. Preliminary studies on the synthesis of alpha 2 macroglobulin by human lymphocytes in vitro. Clin. Exp. Immunol., 1974, 17: 697-701.

192. James K, Tunstall AM, Parker AC, McCormick JN. The association of alpha 2 macroglobulin with lymphocyte membranes in chronic lymphatic leukaemia and other disorders. Clin. Exp. Immunol., 1975, 19: 237-249.

193. Becker C, Harper PC. Alpha 2 macroglobulin on human vascular endothelium. J. Exp. Med., 1976, 144: 1-9.

194. James K, Johnson G, Fundenberg HH. The quantitative estimation of alpha 2 macroglobulin in normal, pathological and cord sera. Clin. Chim. Acta., 1966, 14: 207-214.

195. James K, Merriman J, Gray RS, Duncan LJP, Herd R. Serum alpha 2 macroglobulin levels in diabetes. J. Clin. Pathol., 1980, 33: 163-166.

196. Ganrot PO, Schersten B. Serum alpha 2 macroglobulin concentration and its variation with age and sex. Clin. Chim. Acta., 1967, 15: 113-120.

197. Housley J. Alpha-2 macroglobulin levels in disease in man. J. Clin. Path., 1968, 21: 27-31.

198 McBean LD, Smith JC, Berne BH, Halsted JA. Serum zinc and alpha 2 macroglobulin concentration in myocardial infarction, decubitus ulcer, multiple myeloma, prostatic carcinoma, Down's Syndrome and nephrotic syndrome. Clin. Chim. Acta., 1974, 50: 43-51.

199. Farrow SP, Baar S. The metabolism of alpha 2 macroglobulin in mildly burned patients. Clin. Chim. Acta., 1973, 46: 39-48.

200. Ninneman JL, Fischer JC, Frank HA. Prolonged survival of human skin allografts following thermal injury. Transplantation, 1978, 25: 69-72.

201. Bergqvist D, Nilsson IM. Hereditary alpha-2-macroglobulin deficiency. Scand. J. Haematol., 1979, 23: 433-436.

202. McCormick JN, Nelson D, Tunstall AM, James K. Association of alpha macroglobulins with lymphoid cells. Nature (New Biol.), 1973, 246: 78-81.

203. Healy GM, Parker RC. Cultivation of mammalian cells in defined media with protein and non-protein supplements. J. Cell. Biol., 1966, 30: 539-547.

204. Hovi T, Mosher D, Vaheri A. Cultured human monocytes synthesize and secrete alpha 2 macroglobulin. J. Exp. Med., 1977, 145: 1580-1589.

205. Ford WH, Caspary EA, Shenton B. Purification and properties of a lymphocyte inhibition factor from human serum. Clin. Exp. Immunol., 1973, 15: 169-179.

206. McDaniel MC, Laudico R, Papermaster BW. Association of macrophage-activation factor from a human cultured lymphoid cell line with albumin and alpha 2 macroglobulin. Clin. Immunol. Immunopath., 1976, 5: 91-104.

207. Streilein JS, Hart DA. Serum free culture of hamster lymphoid cells and differential inhibition of lipopolysaccharide stimulation by isologous serum. Infect. Immun., 1976, 14: 463-470.

208. Hubbard WJ. Alpha-2-Macroglobulin enzyme complexes as suppressors of cellular activity. Cell. Immunol., 1978, 39: 388-394.

209. Gallin JI, Kaplan AP. Mononuclear cell chemotactic activity of kallikrein and plasminogen activator and its inhibition by Cl inhibitor and alpha 2 macroglobulin. J. Immunol., 1974, 113: 1928-1934.

210. Dickerson JWT. The role of enteral nutrition in malignant disease. In: Proceedings of the 2nd Congress of European Society of Parenteral and Enteral Nutrition. Eds: Wright PD, Elliott M. Acta Scandinavia, 1981, 422-427.

211. Studley HO. Percentage of weight loss as a basic indicator of surgical risk in patients with chronic peptic ulcer. JAMA, 1936, 106: 458-460.

212. Simms JM, Smith JAR, Woods HF. A modified prognostic index based upon nutritional measurements. Clinical Nutrition, 1982, 1: 71-79.

213. Adler WH, Jones KH, Nariuchi H. Ageing and immune function. In: Recent Advances in Clinical Immunology, vol. 2. Ed: Thompson RA. Churchill Livingstone, Edinburgh, 1980, 77-100.

214. Glasgow AH, Nimberg RB, Menzoian JO et al. Association of anergy with an immunosuppressive peptide fraction in the serum of patients with cancer. N. Eng. J. Med., 1974, 291: 1263-1267.

215. Douglas SD, Faulk WP. Immunological aspects of protein calorie malnutrition. In: Recent Advances in Clinical Immunology, vol. 1. Ed: Thompson RA. Churchill Livingstone, Edinburgh, 1977, 15-39.

216. Meakins JL, Pietsch JB, Bubenick O, et al. Delayed hypersensitivity. Ann. Surg., 1977, 186: 241-250.

217. Francis DMA, Judson RT. Blood transfusion and recurrence of cancer of the colon and rectum. Br. J. Surg., 1987, 74: 26-30.

218. Silverman A, Alexander JC, Potvin C, Chretien PB. Effect of non-rhythmic irradiation on cellular immunocompetence. Surgical Forum, 1975, 26: 345.

219. Copeland EM, MacFadyen BV, Rapp MA, Dudrick SJ. Hyperalimentation and immune competence in cancer. Sugical Forum, 1975, 26: 138-140.

220. Dominioni L, Dionigi R, Dionigi P, et al. Evaluation of possible causes of delayed hypersensitivity impairment in cancer patients. J. Parent. Ent. Nutrition, 1981, 5: 300-306.

221. Dionigi R, Campari M. Nutritional and Immunological abnormalities in malignant disease. In: Proceedings of the 2nd Congress of European Society of Parenteral and Enteral Nutrition. Acta Chirurigica. Scandinavica. Eds: Wright PD, Elliott M., 1981, 435-474.

222. Meakins JL, Christon NV, Shizgal HM, MacLean LD. Therapeutic approaches to anergy in surgical patients. Ann. Surg., 1979, 190: 286-296.

223. Brookes GB, Clifford P. Nutrition status and general immune competence in patients with head and neck cancer. J. Royal Soc. Med., 1981, 74: 132-139.

224. Burke M, Kark AE. Nutritional status and cell mediated immunity in gastro-intestinal cancer. Cinical Oncology, 1980, 6: 372-373.

225. Chandra RK. Immunology of nutritional disorders. Edward Arnold Publishers, London, 1980.

226. Kahan BD. Nutrition and host defence mechanisms. Surg. Clin. N. Amer., 1981, 61: 557-570.

227. Neumann CG, Lawlor GJ, Stiehm ER et al. Immunologic responses in malnourished children. Am. J. Clin. Nutr., 1975, 28: 89-104.

228. McFarlane H. Cell mediated immunity in clinical and experimental protein-calorie malnutrition. In: Malnutrition and the Immune Response. Ed: Suskino RM., Raven Press, New York, 1977, p127.

229. Baker JP, Detsky AS, Wesson DE et al. Nutritional assessment: a comparison of clinical judgement and objective measurements. New Eng. J. Med., 1982, 306: 969-972.

230. Jelliffe DB. The assessment of the nutritional status of the community. WHO Monograph 53, Geneva, WHO, 1966.

231. Bastow MD. Anthropometrics revisited. Proc. Nutr. Soc., 1982, 41: 381-388.

232. Forse RA, Shizgal HM. The assessment of malnutrition. Surgery, 1980, 88: 17-24.

233. Klidjian AM, Foster KJ, Kammerling RM, Cooper A, Karran SJ. Relation of anthropometric and dynamometric variables to serious post-operative complications. Brit. Med. J., 1980, 281: 899-901.

234. Woods HF, Newton DJ, Kay R, Clark RG. The nutritional assessment of hospital patients: a critical review. Acta. Chir. Scand., 1982 Suppl. 507: 171-180.

235. Mullen JL, Buzby GP, Waldman MT, Gertner MH, Hubbs CW, Rosato EF. Prediction of operative morbidity and mortality by pre-operative nutritional assessment. Surgical Forum, 1979, 30: 80-82.

236. Mullen JL, Gertner MH, Buzby GP, Goodhart GL, Rosato EF. Implications of malnutrition in the surgical patient. Archives of Surgery, 1979, 114: 121-125.

237. Harries AD, Jones L, Heatley RV, Rhodes J, Fitzsimons E. Mid arm circumference as simple means of identifying malnutrition in Crohn's disease. Brit. Med. J., 1982, 285: 1317-1318.

238. Grant JP, Custer PB, Thurcow J. Current techniques of nutritional assessment. Surg. Clin. N. Amer., 1981, 61: 437-464.

239. Johnston IDA, Wright PD, Lennard TWJ et al. Malnutrition and cancer. Clin. Oncol., 1981, 7: 83-91.

240. Bistrian BR, Blackburn GL, Sherman M, Scrimshaw NS. Therapeutic index of nutritional depletion in hospitalized patients. Surg. Gynecol. Obstet., 1975, 141: 512-516.

241. Moran L, Custer P, Murphy G. Nutritional assessment of lean body mass. J. Parent. Ent. Nutr., 1980, 4: 595.

242. Williamson DH, Farrell R, Kerr A, Smith R. Muscle-protein catabolism after injury in man, as measured by urinary excretion of 3-methyl-histidine. Clin. Sci. Mod. Med., 1977, 52: 527-533.

243. James WPT. In: Advances in Parenteral Nutrition. Ed: Johnston IDA. MTP Press, Lancaster, 1978, p521.

244. Rich AJ. The assessment of body composition in clinical conditions. Proc. Nutr. Soc., 1982, 41: 389-403.

245. Carpentier YA, Barthel J, Bruyns J. Plasma protein concentration in nutritional assessment. Proc. Nutr. Soc., 1982, 41: 405-417.

246. Irwin TT, Hunt TK. Effect of malnutrition on colonic healing. Ann. Surg., 1974, 180: 765-772.

247. Bozzetti F, Terno G, Longoni C. Parenteral hyperalimentation and wound healing. Surg. Gynecol. Obstet., 1975, 141: 712-714.

248. Mullen JL, Buzby GP, Waldman MT et al. Prediction of operative morbidity and mortality by pre-operative nutritional assessment. Surgical Forum, 1979, 30: 80-82.

249. Reinhardt GF, Myscofski JW, Wilkens DB. Incidence and mortality of hypoalbuminemic patients in hospitalized veterans. J. Parent. Ent. Nutr., 1980, 4: 357-359.

250. Hickman DM, Miller RA, Rombeau JL et al. Serum albumin and body weight as predictors of post-operative course in colorectal cancer. J. Parent. Ent. Nutr., 1980, 4: 314-316.

251. Dionigi R, Cremaschi RC, Jemos V, Dominioni L, Monico R. Nutritional assessment and severity of illness classification systems: a critical review of their clinical relevance. World J. Surg., 1986, 10: 2-11.

252. Solomons NW, Allen LH. The functional assessment of nutritional status: principles, practice and potential. Nutr. Rev., 1983, 41: 33-50.

253. Desai ID, Tavares MLG, Dutra de Oliveira BS et al. Food habits and nutritional status of agricultural migrant workers in Southern Brazil. Am. J. Clin. Nutr., 1980, 33: 702-714.

254. Desai ID, Tavares MLG, Dutra de Oliveira B et al. Anthropometric and cyclo-ergometric assessment of the nutritional status of the children of agricultural migrant workers in Southern Brazil. Am. J. Clin. Nutr., 1981, 34: 1925-1934.

255. Archer TJ, McDonald PJ, Klidjian AM, Karran SJ. Predicting need for nutritional support. Lancet, 1982, 1: 912.

256. Woolfson AMJ. Artificial nutrition in hospital. Br. Med. J., 1983, 281: 1004-1006.

257. Davies CWT, Jones DM, Shearer JR. Hand grip - a simple test for morbidity after fracture of the neck of femur. J. Roy. Soc. Med., 1984, 77: 833-836.

258. Blackburn GL. Discussion paper. Am. J. Surg., 1980, 139: 166-167.

259. Hessov I. Protein energy malnutrition in surgical patients. In: Nutrition and the surgical patient. Ed: Hill GL. Churchill Livingstone, Edinburgh, 1981, 26-38.

260. Ryan JA, Taft DA. Pre-operative nutritional assessment does not predict morbidity and mortality in abdominal operations. Surgical Forum, 1981, 32: 96-98.

261. Brown R, Bancewicz J, Hamid J et al. Failure of delayed hypersensitivity skin testing to predict post-operative sepsis and mortality. Brit. Med. J., 1982, 284: 851-853.

262. Tanner NSB, Clifford P, Carter RL. Immune status and its relation to management and prognosis in squamous carcinoma of the head and neck. Clinical Oncology, 1978, 4: 99-100.

263. Lewis RT, Klein H. Risk factors in post-operative sepsis: significance of pre-operative lymphocytopenia. J. Surg. Research, 1979, 26: 365-371.

264. Cohn SH, Gartenhaus W, Sawitsky A et al. Compartmental body composition of cancer patients by measurement of total body nitrogen, potassium and water. Metabolism, 1980, 30: 222-229.

265. Cohn SH, Gartenhaus W, Vartsky D et al. Body composition and dietary intake in neoplastic disease. Am. J. Clin. Nutr., 1981, 34: 1997-2004.

266. Cohn SH. Body composition of patients with malnutrition and cancer. Clinics in Oncol., 1986, 5: 263-276.

267. Burke M, Bryson EI, Kark AE. Dietary intakes, resting metabolic ratio and body composition in benign and malignant gastro-intestinal disease. Br. Med. J., 1980, 280: 211-215.

268. Carmichael MJ, Clague MB, Keir MJ, Johnston IDA. Whole body protein turnover, synthesis and breakdown in patients with colorectal cancer. Br. J. Surg., 1980, 67: 736-739.

269. Cohn SH, Vartsky D, Vaswani AN et al. Changes in body composition of cancer patients following combined nutritional support. Nutrition and Cancer, 1982, 4: 107-119.

270. Haffejee AA, Angorn IB, Baker LW, Brain PP. Nitrogen balance and the cellular immune response in oesophageal carcinoma. Front. Gastrointest. Res., 1979, 4: 151-157.

271. Law DK, Dudrick SJ, Abdou NI. The effect of dietary protein depletion on immunocompetence. Ann. Surg., 1974, 179: 168-173.

272. Woodruff JF. The influence of quantitated post-weaning undernutrition in Coxsackie virus B3 infection of adult mice. II. Alteration of host defence mechanisms. J. Infect. Disease, 1970, 121: 164-181.

273. Dionigi R, Zonta A, Dominioni L, Gnes F, Ballabio A. The effects of total parenteral nutrition on immunodepression due to malnutrition. Ann. Surg., 1977, 185: 467-474.

274. Dionigi R, Zonta A, Ballabio A. Immunological assessment of malnourished surgical patients undergoing total parenteral nutrition. IRCS Med. Sci., 1975, 3: 424.

275. Law DK, Dudrick SJ, Abdou NI. Immunocompetence of patients with protein calorie malnutrition. Ann. Intern. Med., 1973, 79: 545-550.

276. Copeland EM, MacFadyen BV, Dudrick SJ. Effect of intravenous hyperalimentation on established delayed hypersensitivity in the cancer patient. Ann. Surg., 1976, 184: 60-64.

277. Freeman M, Frankmann C, Beck J, Valdivieso M. Prognostic nutrition factors in lung cancer patients. J. Par. Enter. Nutr., 1982, 6: 122-127.

278. Nixon DW, Moffitt S, Lawson DH et al. Total parenteral nutrition as an adjunct to chemotherapy of metastatic colorectal cancer. Cancer Treat. Rep., 1981, 65: 121-128.

279. Popp MB, Fisher RI, Wesley R et al. A prospective randomized study of adjuvant parenteral nutrition in the treatment of advanced diffuse lymphoma: influence on survival. Surgery, 1981, 90: 195-202.

280. Clamon G, Feld R, Evans WK et al. Effect of hyperalimentation on the survival and response to treatment of patients with small cell lung cancer: a randomized trial. Cancer Treat. Rep., 1985, 69: 167-177.

281. Valdivieso M, Bodey GP, Benjamin RS et al. Role of intravenous hyperalimentation as an adjunct to intensive chemotherapy for small cell bronchogenic carcinoma. Cancer Treat. Rep., 1981, 65 (S5): 145-150.

282. Serrou B, Cupissol D, Plagne R et al. Follow-up of a randomized trial for oat cell carcinoma. Rec. Res. Can. Res., 1982, 80: 246-253.

283. Samuels ML, Selig DE, Ogden S et al . IV hyperalimentation and chemotherapy for Stage II testicular cancer: a randomized study. Cancer Treat. Rep., 1981, 68: 615-627.

284. Shamberger RC, Brennan MF, Goodgame JT et al. A prospective randomized study of adjuvant parenteral nutrition in the treatment of sarcomas: results of metabolic and survival studies. Surgery, 1984, 96: 1-13.

285. Donaldson SS. Nutritional support as an adjunct to radiation therapy. J. Par. Ent. Nutr., 1984, 8: 302-307.

286. Muller JM, Dienst C, Brenner U, Pichlmaier H. Pre-operative parenteral feeding in patients with gastrointestinal carcinoma. Lancet, 1982, 1: 68-71.

287. Holter AR, Rosen HM, Fischer JE. The effects of hyperalimentation on major surgery in patients with malignant disease: a prospective study. Acta. Chir. Scand., 1976, S466: 86-87.

288. Holter AR, Fischer JE. The effects of peri-operative hyperalimentation on complications in patients with carcinoma and weight loss. J. Surg. Res., 1977, 23: 31-34.

289. Heatley RV, Williams RHP, Lewis MH. Pre-operative intravenous feeding - a controlled trial. Post-graduate Med. J., 1979, 55: 541.

290. Moghissi K, Hornshaw J, Teasdale PR, Dawes EA. Parenteral nutrition in carcinoma of the oesophagus treated by surgery. Nitrogen balance and clinical studies. Br. J. Surg., 1977, 64: 125-128.

291. Lim STK, Chou RG, Lam KH et al. Total parenteral nutrition versus gastroscopy in the pre-operative preparation of patients with carcinoma of the oesophagus. Br. J. Surg., 1981, 68: 69-72.

292. Williams RHP, Heatley RV, Lewis MH, Hughes LE. A randomized controlled trial of pre-operative intravenous nutrition in patients with stomach cancer. Br. J. Surg., 1976, 63: 667.

293. Francis DMA, Hoenich N, Ward MK. Evaluation of indwelling right atrial catheter for long-term hemodialysis. In: Progress in artificial organs. Eds: Atsumi K, Maekawa M, Ota K. ISAO Press No.204, Cleveland, 1984, p554-559.

294. Stein TP, Oram-Smith JC, Leskiw MJ, Wallace HW, Miller EE. Tumour-caused changes in host protein synthesis under different dietary situations. Cancer Res., 1976, 36: 3936-3940.

295. White FR. The relationship between underfeeding and tumour formation transplantation and growth in rats and mice. Cancer Res., 1961, 21: 281-286.

296. Popp MB, Wagner SC, Brito OJ. Host and tumour responses to increasing levels of intravenous nutritional support. Surgery, 1983, 92: 300-307.

297. Nixon DW. Ability of enteral and parenteral nutritional supplementation to replete the wasted host. Clinics in Oncology, 1986, 5: 355-364.

298. Jeejeebhoy KN. Intravenous nutrition - an overview. In: Nutrition and the surgical patient. Ed: Hill GL. Churchill Livingstone, Edinburgh, 1981, 118-132.

299. MacFie J, Smith RC, Hill GL. Glucose or fat as a non-protein energy source? A controlled clinical trial. Gastroenterology, 1981, 80: 103-107.

300. Mertin J, Hughes D. Specific inhibitory action of polyunsaturated fatty acids on lymphocyte transformation induced by PHA and PPD. Int. Archs. Allergy Appl. Immunol., 1975, 48: 203-210.

301. Mertin J, Shenton BK, Field EJ. Unsaturated fatty acids in multiple sclerosis. Brit. Med. J., 1973, 2: 777-778.

302. Offner H, Clausen J. Inhibition of lymphocyte response to stimulants induced by unsaturated fatty acids and prostaglandins. Lancet, 1974, 2: 400-401.

303. Ring J, Seiffert J, Mertin J, Brendel W. Prolongation of skin allografts in rats by treatment with linoleic acid. Lancet, 1974, 2: 1331.

304. Kort WJ, Weijma IM, Westbroek DL. Effects of stress and dietary fatty acids on allograft survival in the rat. Eur. Surg. Res., 1979, 11: 434-444.

305. Mertin J. Unsaturated fatty acids and renal transplantation. Lancet, 1974, 2: 717.

306. Udall PR, Wilkinson R, McHugh MI et al. Unsaturated fatty acids and renal transplantation. Lancet, 1974, 2: 514.

307. Harmer M. The case for TNM. Clin. Oncology, 1971, 3: 131-135.

308. Holst PM. Studies on the effects of tuberculin. Tubercle (Edinburgh), 1922, 3: 249-256.

309. Rich AR, Lewis MR. The nature of allergy in tuberculosis as revealed by tissue culture studies. Bull. Johns Hopkins Hosp., 1932, 50: 115-131.

310. David JR, Al-Askari S, Lawrence HS, Thomas L. Delayed hypersensitivity in vitro. 1. The specificity of inhibition of cell migration by antigens. J. Immunology, 1964, 93: 264-273.

311. Bloom BR, Bennett B. Mechanisms of a reaction in vitro associated with delayed-type hypersensitivity. Science, 1966, 153: 80-82.

312. Diengdoh JV, Turk JL. Electrophoretic mobility of guinea pig peritoneal exudate cells in hypersensitivity reactions. Int. Arch. Allergy, 1968, 34: 297-302.

313. Reuss F. Memoires de la Societe des Naturalistes de Moscou. 1809, 2: 337.

314. Helmholtz H. Ann. Phys. Chem. (Ullidemannis Ann). 1879, 7: 337-382.

315. Smoluchowski M. Anz-Akad. Wiss. Krakau, 1903, 3: 182-199.

316. Sundaram K, Phondke GP, Sundaresan P. In vitro studies on antibody production by lymph node cells using cell electrophoresis. Immunology, 1967, 13: 433-439.

317. Sachtleben P. The influence of antibodies on the electrophoretic mobility of red cells. In: Cell Electrophoresis. Ed: Ambrose EJ. 1964, Churchill, London.

318. Forrester JA, Dumonde DC, Ambrose EJ. The effect of antibody on cells. II. Changes in electrophoretic mobility of ascites tumour cells treated with antibodies and complement. Immunology, 1965, 8: 37-48.

319. Sachtleben P, Ruhenstoth-Bauer G. The change in the electrical surface charge of erythrocytes by agglutinating and sensitising substances. Med. Exp., 1962, 6: 183-192.

320. Field EJ, Caspary EA. Demonstration of sensitized lymphocytes in blood. J. Clin. Path., 1971, 24: 179-181.

321. Mertin J, Hughes D, Shenton BK, Dickinson JP. In vitro inhibition by unsaturated fatty acids of the PPD and PHA-induced lymphocyte response. Klin. Wschr., 1974, 52: 248-250.

322. Hughes D, Caspary EA, Field EJ. Delayed hypersensitivity studies in experimental allergic encephalomyelitis; a comparison of macrophage migration inhibition tests and electrophoretic mobility. Z. Immun. Forsth., 1970, 141: 14-19.

323. Preece AW, Light PA. The macrophage electrophoretic mobility (MEM) test for malignant disease. Further clinical investigations and studies on macrophage slowing factors. Clin. Exp. Immunol., 1974, 18: 543-552.

324. Pritchard JAV, Moore JL, Sutherland WH, Joslin CAF. Technical aspects of the macrophage electrophoretic mobility (MEM) test for malignant disease. Br. J. Cancer, 1973, 28: Suppl. 1. 229-236.

325. Pritchard JAV, Moore JL, Sutherland WH, Joslin CAF. Evaluation and development of the macrophage electrophoretic mobility (MEM) test for malignant disease. Br. J. Cancer, 1973, 27: 1-9.

326. Porzsolt FR, Tantz CH, Ax W. In Behring Inst. Mitt., 1975, No.57, 128 (quoted by Pritchard(324)).

327. Shenton BK, Jenssen HL, Werner H, Field CJ. A comparison of the kinetics of the macrophage electrophoretic mobility (MEM) and the tanned sheep erythrocyte electrophoretic mobility (TEEM) tests. J. Immun. Methods, 1977, 14: 123-139.

328. Donnelly PK, Shenton BK, Veitch PS et al. A rapid laboratory test to predict the likelihood of early rejection in kidney transplantation. European Surgical Research, 1982, 14: 79-80.

329. Field EJ, Caspary EA. Lymphocyte sensitisation: an in vitro test for cancer? Lancet, 1970, 2: 1337-1341.

330. Caspary EA, Field EJ. Specific lymphocyte sensitization in cancer. Is there a common antigen in human malignant neoplasia? Brit. Med. J., 1971, 2: 613-617.

331. Field EJ, Caspary EA, Smith KS. Macrophage electrophoretic mobility (MEM) test in cancer: a critical evaluation. Br. J. Cancer, 1973, 28: Suppl. 1, 208-214.

332. Goldstone AH, Kerr L, Irvine WJ. The macrophage electrophoretic migration test in cancer. Clin. Exp. Immunol., 1973, 14: 469-472.

333. Rawlins GA, Wood JMF, Bagshawe KD. Macrophage electrophoretic mobility (MEM) with myelin basic protein. Br. J. Cancer, 1976, 34: 613-618.

334. Lampert F, Nitzschke U, Zwerge T. Lymphocyte sensitization in childhood solid tumours and lymphoblastic leukaemia, measured by electrophoretic mobility test. Br. J. Cancer, 1977, 35: 844-849.

335. Meyer-Reinecker H, Jenssen HL, Kohler H. Zur Bedenting des Makrophagen elektrophorese mobilitatstests fue die diagnostik der geschwulte des zentralnervensystems. Dtsch. Med. Wochenschr., 1975, 100: 538-543.

336. Muller M, Irmscher J, Fischer R. Immunological tumour profile: organ specific carcinoma diagnosis in patients employing the macrophage electrophoretic mobility test. Cancer Letters, 1977, 2: 139-146.

337. Tognella S, Mantovani G, Floris C. Test citoferometrico per l'evidenzienzione di linfociti sensibilizzati ad antigeni neoplastici (MEM test). Tumori, 1974, 60: 203-210.

338. Nakajima T, Gonmori M, Isojima K et al. In vivo and in vitro cell mediated immune reactions to autochthonous tumour extracts in patients with gastric cancer. Gann., 1976, 67: 537-547.

339. Shaw A, Ettin G, McPherson TA. Responses of cancer patients in the MEM test: not just a function of charge on basic proteins. Br. J. Cancer, 1976, 34: 7-13.

340. Glaves D, Harlos JP, Weiss L. The macrophage electrophoretic mobility test: results on carcinoma of the colon and rectum. Int. J. Cancer, 1977, 19: 474-481.

341. Shenton BK, Proud G, Veitch PS, Taylor RMR. The predictive value of pre transplant mixed lymphocyte reaction in renal transplantation. A prospective six year study. In: Cell electrophoresis: Clinical application and methodology. Eds: Preece AW, Savolovic D. Elsevier/North Holland Biomedical Press, 1979, 91-100.

342. Proud G, Shenton BK, Smith BM. Blood transfusion and renal transplantation. Brit. J. Surgery, 1979, 66: 678-682.

343. Preece AW, Light PA. Developments in cancer research. Vol. 6. Cell electrophoresis in cancer and other clinical research. Elsevier/North Holland Biomedical Press, Amsterdam, 1981.

344. Shenton BK, Francis DMA, Veitch PS. Electrophoretic tests of lymphocyte function - clinical applications. In: Cell electrophoresis in cancer and other clinical research. Eds: Preece AW, Light PA. Elsevier/North Holland Biomedical Press, 1981, 99-114.

345. Boyum A. Separation of leucocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest., 1968, Suppl. 97, 21: 107-109.

346. Lymphoprep Data Sheet, 1977, supplied by Nyegaard and Co., Postboks 4220, Torshov, Oslo 4, Norway.

347. Roitt I. Hypersensitivity. In: Essential Immunology. Blackwell Scientific Publications, Oxford, 4th Ed., 1980, p243.

348. Stavitsky AB. Micromethods for the study of proteins and antibodies. J. Immunol., 1953, 72: 360-367.

349. Shenton BK, Smith BM. The tanned erythrocyte electrophoretic mobility (TEEM) test for cellular sensitisation: optimal preparation and properties of the indicator cell population. IRCS Med. Sci., 1977, 5: 445.

350. Dewar PJ, Murray S. Lymphocytotoxic antibody detection and cross matching for renal transplantation. Transplantation, 1976, 21: 387-390.

351. Laurell C-B. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Analytical Biochemistry, 1966, 15: 45-52.

352. Creighton WD, Lambert PH, Miescher PA. Detection of antibodies and soluble antigen-antibody complexes by precipitation with polyethylene glycol. J. Immunol., 1973, 111: 1219-1227.

353. Digeon M, Laver M, Riza J, Bach JF. Detection of circulating immune complexes in human sera by simplified assays with polyethylene glycol. J. Immunol. Meth., 1977, 16: 165-183.

354. D'Amelio R, Bilotta P, Pachi A, Aiuti F. Circulating immune complexes in normal pregnant women and in some conditions complicating pregnancy. Clin. Exp. Immunol., 1979, 37: 33-37.

355. Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, 1965, 2: 235-254.

356. Mann HB, Whitney DR. On a test of whether one of two random variables is stochastically larger than the other. Ann. Math. Statist., 1947, 18: 50-60.

357. Wilcoxon F. Individual comparisons by ranking methods. Biometrics Bull., 1945, 1: 80-83.

358. Swinscow TDV. Statistics at square one. 4th Ed. British Medical Association Publications, London, 1978, 33-70.

359. Zubler RH, Lambert PH. Immune complexes in clinical investigation. In: Recent Advances in Clinical Immunology. Ed: Thompson RA. Churchill Livingstone, Edinburgh, 1977, 1: 125-147.

360. Dainow IL. Safety of Intralipid. Lancet, 1980, 2: 1020.

361. MacFadyen BV, Dudrick SJ, Tagudar EP et al. Triglyceride and free fatty acid clearances in patients receiving complete parenteral nutrition using a ten per cent soybean oil evaluation. Surg. Gyne. Obst., 1973, 137: 813-815.

362. Ota DM, Copeland EM, Corriere JN et al. The effect of a 10% soybean oil emulsion on lymphocyte transformation. J. Parent. Ent. Nutr., 1978, 2: 112-115.

363. Freund U, Krausz Y, Levij IS. Iatrogenic lipidosis following prolonged intravenous hyperalimentation. Am. J. Clin. Nutr., 1975, 28: 1156-1160.

364. Jarstrand C, Berghem L, Lahnborg G. Human granulocyte and reticuloendothelial system function during Intralipid infusion. J. Parent. Ent. Nutr., 1978, 2: 663-670.

365. Hallberg D. Studies on the elimination of exogenous lipids from the blood stream. Acta Physiol. Scand., 1965, 64: 299-305.

366. Zittel RS, Scholler KL, Oehlert W. Munch. Med. Wochenschr., 1967, 109, 349. (quoted by MacFadyen et al(361)).

367. Hambraeus L. Metabolism of carbohydrates, fats and proteins. In: Scientific Foundations of Surgery, 2nd Ed. Eds: Wells C, Kyle J, Dunphy JE. William Heinemann Medical Books Ltd., London, 1974, 577-591.

368. Baldwin RW, Pimm MV. BCG immunotherapy of a rat sarcoma. Brit. J. Cancer, 1973, 28: 281-287.

369. Perri GC, Faulk M, Shapiro E et al. Function of the thymus and growth of tumour homograft. Nature, 1963, 200: 1294-1296.

370. Attia MAM, Weiss D. Immunology of spontaneous mammary carcinomas in mice. Cancer Res., 1966, 26: 1787-1800.

371. Gaugas JM. Glycoproteins in pregnancy serum which interact with concanavalin-A and may suppress lymphocyte transformation. Transplantation, 1974, 18: 538-541.

372. Riggio RR, Schwartz GH, Bull FG, Stenzel KH, Rubin AL. Alpha-2 globulins in renal graft rejection. Transplantation, 1969, 8: 689-694.

373. Davis JC, Finn R, Hipkin LJ, St. Hill CA. Do plasma glycoproteins induce lymphocyte hyporesponsiveness and insulin resistance? Lancet, 1978, 2: 1343-1345.

374. Proud G. Blood transfusion and organ transplantation. Ann. R. Coll. Surg., 1980, 62: 271-279.

375. Lenhard V, Massen G, Seifert P, Johannsen R, Grosse-Wilde H. Characterisation of transfusion-induced suppressor cells in prospective kidney allograft recipients. Transplant. Proc., 1982, 14: 329-332.

376. Marquet RL, Heystek GA, Niessen GJCM, Jeekel J. Induction of suppressor cells by a single blood transfusion in rats. Transplant. Proc., 1982, 14: 397-399.

377. Kerman RH, Van Buren CT, Payne W et al. Influence of blood transfusions on immune responsiveness. Transplant. Proc., 1982, 14: 335-337.

378. Singal DP, Joseph S, Szewczuk MR. Possible mechanism of the beneficial effect of pre-transplant blood transfusions on renal allograft survival in man. Transplant. Proc., 1982, 14: 316-318.

379. Keown PA, Descamps B. Improved renal allograft survival after blood transfusion: a non-specific, erythrocyte-mediated immunoregulatory process. Lancet, 1979, 1: 20-22.

380. Van der Linden CJ, Burman WA, Vegt PA. In: Opelz G, Persijn GG. Blood transfusion in renal transplantation. Transplant. Proc., 1981, 13: 1685-1689.

381. Benzonna O, Carpenter N, Jeannet M et al. Circulating immune complexes in dialysed patients waiting for renal transplantation. Transplant. Proc., 1981, 13: 1556-1558.

382. Francis DMA, Shenton BK, Proud G, Taylor RMR. Tumor growth and blood transfusion. J. Exp. Clin. Cancer Res., 1982, 1: 121-126.

383. Alomran A, Shenton BK, Proud G, Francis DMA, Donnelly PK, Taylor RMR. Possible mechanism of immune regulation produced by alph-2-macroglobulin. Lancet, 1982, 2: 1168.

384. Gascon P, Zoumbos NC, Young NS. Immunologic abnormalities in patients receiving multiple blood transfusions. Ann. Intern. Med., 1984, 100: 173-177.

385. Macleod AM, Mason RJ, Stewart KN et al. Fc-receptor-blocking antibodies develop after blood transfusions and correlate with good graft outcome. Transplant. Proc., 1983, 15: 1019-1021.

386. Singal DP, Joseph S, Ludwin D. Blood transfusions, suppressor cells and anti-idiotypic antibodies. Transplant. Proc., 1985, 17: 1104-1107.

387. Ettenger RB, Jordan SC, Arnett J, Robinson BJ, Fine RN. Specific anti-donor lymphocytotoxic antibodies following blood transfusion from non-related donors. Transplant. Proc., 1982, 14: 347-348.

388. Van Rood JJ. Pretransplant blood transfusion: Sure! But how and why? Transplant. Proc., 1983, 15: 915-916.

389. Donnelly PK, Shenton BK, Alomran AM, Francis DMA, Proud G, Taylor RMR. A new mechanism of humoral immunodepression in chronic renal failure and its importance to dialysis and transplantation. Proc. EDTA, 1983, 20: 297-304.

390. Francis DMA, Shenton BK. Blood transfusion and tumour growth: evidence from laboratory animals. Lancet, 1981, 2: 871.

391. Francis DMA, Buren C, Clunie GJA. Acceleration of B16 melanoma growth in mice following blood transfusion. Surgery, 1987, 102: 485-492.

392. Stimson WH. Pregnancy-associated alpha-2-glycoprotein and cancer. J. Roy. Coll. Surg. Edin., 1978, 23: 253-259.

393. Roth JA. Detection of a suppressive immunoregulatory factor (IRF) in the sera of sarcoma patients by enzyme-linked immuno assay (ELISA) and correlation with clinical course. J. Surg. Oncol., 1983, 23: 273-281.

394. Baldwin RW, Byers VS, Robins RA. Immune complexes in cancer. In: Tumour Markers: Impact and prospects. Eds: Boelsma E, Rumke P. Elsevier/North Holland Biomedical Press, 1979, 151-165.

395. Namer M, Krebs B, Lupo R, Schneider M, Lalanne C. Plasma CEA level in relationship with hormone therapy, chemotherapy and hormono-chemotherapy in breast cancer. In: Cancer related antigens. Ed: Franchimont P. North Holland Publishing Company, 1976, 189-198.

396. Bagshawe KD. Immunodiagnosis of cancer. In: Cell electrophoresis in cancer and other clinical research. Eds: Preece AW, Light PA. Elsevier/North Holland Biomedical Press, 1981, 17-23.

397. Cooper EH, Turner R, Milford-Ward A, Neville AM. Multiparametric tests in the monitoring of cancer. In: Cancer related antigens. Ed: Franchimont P. North Holland Publishing Company, 1976.

398. Shenton BK, Donnelly PK, Friedman P et al. Rapid in vitro assessment of lymphocyte dose response to cyclosporin A. Submitted for presentation to the Surgical Research Society, May 1983.

399. Leapman SB, Fico RS, Smith EJ. Differential effects of cyclosporin A. Transplant. Proc., 1981, 13: 405-409.

400. Metropolitan Life Insurance Company. New weight standards for men and women. Metropolitan Statistical Bulletin 1959, 40: 1-4.

401. Keys A. Is there an ideal body weight? Br. Med. J., 1986, 293: 1023-1024.

402. Seltzer CC. Limitations of height-weight standards. N. Eng. J. Med., 1965, 272: 1132.

403. Editorial. Appraisal of nutrition. N. Eng. J. Med., 1965, 272: 1129.

404. Bistrian B. Assessment of protein energy malnutrition in surgical patients. In: Nutrition and the surgical patient. Ed: Hill GL. Churchill Livingstone, Edinburgh, 1981, 39-54.

405. Bistrian BR, Sherman M, Blackburn GL, Marshall R, Shaw C. Cellular immunity in adult marasmus. Arch. Int. Med., 1977, 137: 1408-1411.

406. Starzl T. Experience in hepatic transplantation. W.B. Saunders Co., Philadelphia, 1969, 350-373.

407. Arai S, Yamamoto H, Itoh K, Kumagai K. Suppressive effect of human natural killer cells on pokeweed mitogen induced B cell differentiation. J. Immunol., 1983, 131: 651-657.

408. Katz P, Mitchell SR, Cupps TR, Whalen G. Modulation of B lymphocyte responses by natural killer cells. Fed. Proc., 1986, 45: 256.

PUBLICATIONS RELATED TO THE WORK DESCRIBED IN THIS THESIS

1. Francis DMA, Shenton BK, Proud G, Veitch PS, Taylor RMR. Immunosuppressive activity in the plasma of patients with malignant disease. Br. J. Surg., 1980, 67: 820 (abstract)

2. Johnston IDA, Wright PD, Lennard TWJ, Clague MB, Carmichael MJ, Francis DMA, Williams RPH. Malnutrition and cancer. Clinical Oncology, 1981, 7: 83-91

3. Shenton BK, Francis DMA, Veitch PS. Electrophoretic tests of lymphocyte function - clinical applications. In: "Cell electrophoresis in cancer and other clinical research". Eds: Preece AW, Light PA. Elsevier, North Holland Biomedical Press, 1981, 99-114

4. Francis DMA, Shenton BK. Blood transfusion and tumour growth: evidence from laboratory animals. Lancet, 1981, 2: 871

5. Francis DMA, Shenton BK, Johnston IDA. Immunosuppressive factors in the plasma of malnourished hospital patients. J. Parenteral & Enteral Nut., 1981, 5 (4): 352 (abstract)

6. Francis DMA, Shenton BK, Rogaly E, Johnston IDA. The effects on lymphocyte function of solutions used for parenteral nutrition. J. Parenteral & Enteral Nut., 1981, 5 (4): 353 (abstract)

7. Francis DMA, Shenton BK, Johnston IDA. Intravenous fluids and lymphocyte function. Brit. J. Intravenous Therapy, 1982, 3 (10): 5-10

8. Alomran A, Shenton BK, Proud G, Francis DMA, Donnelly PK, Hubbard WJ, Taylor RMR. Possible mechanism of immune regulation produced by a-2-macroglobulin. Lancet, 1982, 2: 1168

9. Francis DMA, Shenton BK, Proud G, Taylor RMR. Tumour growth and blood transfusion. J. Exp. Clin. Cancer Res., 1982, 1: 121-126

10. Donnelly PK, Shenton BK, Alomran A, Francis DMA, Proud G, Taylor RMR. The clinical relevance of alpha-2-macroglobulin. Ann. N.Y. Acad. Sci., 1983, 412: 382-387

11. Alomran A, Shenton BK, Donnelly PK, Francis DMA, Proud G, Taylor RMR. A possible mechanism of immunoregulation produced by alpha-2-macroglobulin. Ann. N.Y. Acad. Sci., 1983, 421: 394-397

12. Alomran A, Shenton BK, Donnelly PK, Francis DMA, Proud G, Taylor RMR. Isolation and characterisation of suppressive peptide from alpha-2-macroglobulin protease interaction. In: Electrophoresis '83. Ed: Hirai H. Walter de Gruyter & Co., Berlin, 1984, 387-390

13. Alomran A, Shenton BK, Francis DMA, Veitch P, Proud G, Taylor RMR. Agarose isoelectric focusing: a preparative technique for studying the immunosuppressive property of alpha-2-macroglobulin. In: Electrophoresis '83. Ed: Hirai H. Walter de Gruyter & Co., Berlin, 1984, 589-592

14. Francis DMA, Shenton BK, Proud G, Taylor RMR. Immunosuppressive plasma factors in malignant disease. Aust. N.Z. J. Surg., 1985, 55: 111-120

15. Francis DMA, Shenton BK. Sequential measurements of plasma lymphocyte suppressive activity in rats bearing methylcholanthrene-induced sarcomas. J. Exp. Clin. Cancer Res., 1986, 5: 33-38

16. Francis DMA, Shenton BK. Fat emulsion adversely affects lymphocyte reactivity. Aust. N.Z. J. Surg., 1987, 57: 323-329